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The Mycoplasmas

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INTRODUCTION

The mycoplasmas are the smallest and simplest self-replicating procaryotes. The mycoplasma cell contains only the minimum set of organelles essential for cell growth and replication: a plasma membrane to separate the cytoplasm from the external environment, ribosomes to assemble the cell proteins, and a double-stranded deoxyribonucleic acid (DNA) molecule to provide the information for protein synthesis. Unlike all other procaryotes, mycoplasmas have no cell wall. The cell biology of these organisms is interesting not only to mycoplasmologists but also to the many workers who use mycoplasmas as simple model systems for studying general biological problems, particularly those concerning membrane structure and function.

The information explosion has been quite pronounced in mycoplasmology, as the discoveries of insect and plant mycoplasmas and of mycoplasma viruses in the early 1970s have attracted many new workers from different disciplines. It is impossible to present a complete and well-balanced picture of the cell biology of mycoplasmas in a single review of a reasonable size. Hence, this review will focus on new developments since the last reviews on physiology and cell biology of mycoplasmas were written in 1972 (182, 236). For a complete and systematic treatment of all aspects of mycoplasmology, the reader is referred to a two-volume treatise to be published early in 1979 (M. F. Barile, S. Razin, J. G. Tully, and R. F. Whitcomb, ed., *The Mycoplasmas*). Proceedings of several meetings and symposia held during the past 5 years on various aspects of mycoplasmology also contain a wealth of information (21, 76, 186).

ECOLOGY

The scope of mycoplasmology has been considerably widened during the last few years. Not only has the total number of species been brought to over 60 (329), but several wall-less procaryotes resembling the classical animal mycoplasmas have been recently isolated from insects, plants, and peculiar ecological niches, such as hot and high-acidic coal refuse piles and rumens of cattle and sheep (Table 1). We are, however, still far from having cultivated and identified all the wall-less procaryotes. Mycoplasmas have been cultivated from only a few diseased plants and insects, though microscopical evidence indicates their presence in many more (21). Moreover, electron microscopy suggests the presence of wall-less procaryotes resembling mycoplasmas in organisms as diverse as amphibians (10), mollusks (117), trematode worms (210), and fungi (119, 259). Although identifying an organism as a mycoplasma requires its cultivation, experience with insect and plant mycoplasmas indicates that tentative identification on the basis of morphological evidence alone should not be discounted. The decision made in 1967 to separate the wall-less procaryotes from eubacteria into a new class, *Mollicutes,* has in retrospect proven to be a very wise one, since the class status reflects the phylogenetic diversity of the mycoplasmas and enables classification within a single taxonomic unit of organisms genetically very remote from each other. The trivial name mycoplasmas will be used in this review rather loosely to denote any species included in the class *Mollicutes,* whereas the trivial name acholeplasmas, ureaplasmas, anaeroplasmas, spiroplasmas, or thermoplasmas will be used when reference is made to members of the corresponding genus, rather than to a defined species within the genus.

Insect and Plant Mycoplasmas

The most exciting developments in mycoplasmology during the past 6 years or so have been

TABLE 1. *Taxonomy and properties of organisms included in the class Mollicutes*[a]

Classification	Current no. of recognized species	Genome		Cholesterol requirement	NADH oxidase localization	Characteristic properties	Habitat
		Size ($\times 10^8$ daltons)	G+C content (%)				
Mycoplasmataceae							
Mycoplasma	ca. 50	5	23–41	+	Cytoplasm		Animals
Ureaplasma	1	5	28	+	ND	Urease activity	Animals
Acholeplasmataceae							
Acholeplasma	6	10	29–35	−	Membrane		Animals
Spiroplasmataceae							
Spiroplasma	1	10	26	+	Cytoplasm	Helical filaments	Insects and plants
Genera of uncertain taxonomic position							
Anaeroplasma	2	ND	29–34	Some + Some −	ND	Anaerobic; some digest bacteria	Rumens of cattle and sheep
Thermoplasma	1	10	46	−	Membrane	Thermophilic (optimum 59°C) and acidophilic (optimum pH 1.0–2.0)	Burning coal refuse piles

[a] For references, see Tully and Razin (329). Abbreviations: G+C, guanine plus cytosine; NADH, reduced nicotinamide adenine dinucleotide; ND, not determined.

in the field of insect and plant mycoplasmas. The cultivation in vitro of the first plant mycoplasma, that causing the "stubborn" disease in citrus, signified a major breakthrough (274) after more than 5 years of relentless efforts to cultivate the mycoplasma-like organisms detected within sections of phloem from a wide variety of plants suffering from yellows diseases (64, 187). The critical factor enabling the successful cultivation of the stubborn disease agent was the raising of the osmolarity of the growth medium to resemble that in the plant by adding high concentrations of sorbitol and other sugars (274, 275). The successful cultivation of the first plant-infecting mycoplasma occurred about the same time as the discovery that plant and insect mycoplasmas resemble spirochetes in forming helical motile filaments (66). The helical shape of the filaments inspired the trivial name spiroplasma (65), later used as the generic name of the stubborn disease agent, *Spiroplasma citri* (275). More than 3 years elapsed before the second plant mycoplasma, that causing corn stunt disease, was cultivated in vitro (40, 354). Chen and Liao (40) succeeded in this by increasing the osmolarity of the medium of Chen and Granados (39), previously found suitable only for maintaining the corn stunt organism, while Williamson and Whitcomb (354) succeeded in cultivating the same spiroplasma in a medium incorporating that used for *Drosophila* cell cultures with that used for cultivating *S. citri*.

A third spiroplasma, more recently cultivated (331), is the suckling mouse cataract agent. Originally isolated from ticks and believed to be a spirochete (44), it is the first spiroplasma found capable of infecting and causing disease in animals (332). Since the suckling mouse cataract agent failed to grow in the growth media of *S. citri* and the corn stunt organism, a more complex medium, containing, among other components, α-ketoglutaric acid, was devised (331). To date, four more spiroplasmas, causing diseases in the honeybee (63), the cactus *Opuntia tuna* (157), Bermuda grass (T. A. Chen and H. C. Su, personal communication), and rice (T. A. Chen and J. C. Chiu, personal communication), have been cultivated, but no details are as yet available concerning the culture media. In all cases of successful cultivation, Koch's postulates were fulfilled. Although the compounding of growth media for spiroplasmas is still largely empirical, the major obstacles appear to have been overcome, and we can expect the cultivation of more insect and plant spiroplasmas in the very near future.

Although most of the isolated spiroplasmas cause disease in plants, insects are probably their primary hosts. In fact, the sex ratio organism, a spiroplasma causing elimination of males in *Drosophila*, and the suckling mouse cataract agent have been isolated only from insects and are not known to infect plants (30, 44, 345). The brain damage and cataract produced by intracerebral inoculation of suckling mice and rats with the suckling mouse cataract agent as well as the pathogenicity of this spiroplasma in embryonated eggs might be only manifestations of laboratory-induced infection, as there is no evidence for suckling mouse cataract agent infecting animals in nature (332). The low host specificity exhibited by some animal mycoplasmas

(236) is even more pronounced in spiroplasmas. *S. citri* could not only infect a large number of different insect species in the laboratory, but it was also isolated in nature from different species of leafhoppers known to feed on plants other than citrus (35, 233). In the laboratory, the corn stunt spiroplasma could even infect and reproduce in *Drosophila* species, though this infection, unlike that with the natural *Drosophila* pathogen, the sex ratio organism spiroplasma, could not be transmitted transovarially and did not eliminate the male population (353, 354). The ease with which spiroplasmas can infect insects may be attributed to the primitive immunological responses and inefficient defense mechanisms of insects, which allow infection by even animal mycoplasmas. The fact that Whitcomb et al. (344) succeeded in infecting leafhoppers with *Acholeplasma laidlawii* and *Acholeplasma granularum* and the finding that ticks feeding on cattle suffering from contagious bovine pleuropneumonia harbored *Mycoplasma mycoides* subsp. *mycoides* (292) suggest that insect reservoirs may comprise part of the natural ecology of animal mycoplasmas. Another interesting point is that spiroplasmas frequently caused damage to the insects expressed for the most part by a reduced life span. This is unusual, since pathogens lethal to the insects transmitting them are rare (345, 346, 354).

The low host specificity exhibited by spiroplasmas when infecting insects is no less striking when different plants are infected with the same spiroplasma. Leafhoppers carrying *S. citri* infected a variety of plants other than citrus, including clover, *Vinca rosea*, and *Vicia faba*, and produced disease with symptoms of the classical yellows diseases; *S. citri* could be reisolated from the diseased plants (35, 56, 189, 233). These findings raise several basic questions. Does the low host specificity reflect inefficient plant defense mechanisms, or does it express a close phylogenetic relatedness among the different spiroplasmas? Does the production of yellows disease symptoms in different plants by *S. citri* imply that the mycoplasma-like bodies observed in numerous plants suffering from yellows disease are in fact spiroplasmas related to *S. citri* but differing in some in vitro nutritional requirements? The available information is insufficient to clearly answer the above questions. Failure to demonstrate helical filaments in plant tissues infected with mycoplasma-like organisms does not necessarily indicate that the infecting organisms are not spiroplasmas, as helicity can be a transitory feature and may even totally disappear (see Cell Morphology and Replication). In any case, all the mycoplasmas with classical mycoplasma morphology isolated from plants were established animal *Mycoplasma* and *Acholeplasma* species (236) and may thus be regarded as contaminants or secondary invaders, whereas all the bona fide new species isolated from plants and insects were spiroplasmas.

The classification of the newly isolated spiroplasma strains is problematic. Although the peculiar characteristics of the spiroplasmas warrant their being placed in a separate family, *Spiroplasmataceae* (Table 1), the family contains only one established species, *S. citri*, so far. All the other cultivable spiroplasmas have not been given binomial names as yet, despite their having been isolated from different plant and insect hosts and their different nutritional requirements. The main reason for not naming them is the considerable antigen sharing among the spiroplasmas, as was demonstrated by several serological techniques, including the highly specific growth inhibition test (329, 331, 345, 352-354). Serology has been of such importance in distinguishing classical mycoplasma species that Freundt et al. (E. A. Freundt, H. Ernø, and R. M. Lemcke, *in* J. R. Norris and D. W. Gibbons, ed., *Methods in Microbiology*, vol. 10, in press) define a mycoplasma species as a group of strains so closely related that an antibody produced against one strain will prevent replication and metabolism in all other strains within that group. In the case of spiroplasmas, this principle obviously does not hold true. Thus, more weight should be placed on DNA hybridization tests and electrophoretic comparison of cell proteins, ribosomal ribonucleic acids (rRNA's), and DNA fragments (see Genome and Ribosomal and Transfer Ribonucleic Acids). The few electrophoretic data available show almost identical protein profiles among strains of *S. citri* (275) and differences between *S. citri*, the new honeybee spiroplasma (63), and the *Opuntia* spiroplasma (157). A collaborative study on the base composition and hybridization of DNAs from the cultivable spiroplasmas is underway (J. G. Tully, personal communication). A priori, it is unlikely that all these spiroplasmas, causing such different diseases in different insects, plants, and possibly animals, belong to only one species.

Anaeroplasmas and Thermoplasmas

Of the animal mycoplasmas recently isolated, the obligate anaerobic mycoplasmas in the rumens of cattle and sheep (254, 255) are physiologically the most interesting. In their cytology and DNA base composition and in the cholesterol requirement exhibited by some strains, they resemble the classical mycoplasmas. Nevertheless, their absolute requirement for anaerobic conditions justifies their inclusion in a new ge-

nus, *Anaeroplasma*. Some of the anaeroplasmas have the ability, peculiar among mycoplasmas, of digesting bacteria and have accordingly been named *Anaeroplasma bactoclasticum* (254, 255). Very different and phylogenetically quite remote from all the parasitic mycoplasmas are the wall-less procaryotes isolated by Darland et al. (58) from self-heated coal refuse piles. Though their lack of cell walls justifies their inclusion in the class *Mollicutes*, their motility by flagella, peculiar DNA, RNA, and lipids, minimal nutritional requirements, and complex electron transport system (see appropriate sections) set them apart from all other members of the *Mollicutes*. Moreover, they are the only nonparasitic members in this class. The ecological niche of these organisms is unique: burning coal refuse piles, where the organisms can find the extreme conditions to which they are adapted—high temperature (59°C optimum) and extremely low pH (1.0 to 2.0 optimum)—hence the name *Thermoplasma acidophilum*. Attempts to isolate thermoplasmas from acidic hot springs have failed, which presents an interesting ecological dilemma. Coal refuse piles are man-made and of relatively recent origin; thus, it is doubtful whether they are the primary and natural habitats of thermoplasmas (12, 19). Cell protein profiles indicate a high degree of homogeneity among strains of *T. acidophilum* collected at different locations, though serological tests suggest the presence of at least five different serological groups (12, 20).

The question of whether *T. acidophilum* is a true *Mollicute* or a stable L-phase of a thermophilic and acidophilic bacterium, such as *Sulfolobus acidocaldarius* (72, 204), is unresolved. *Sulfolobus* resembles the *Thermoplasma* in lacking peptidoglycan, but it is distinguishable from the spherical *Thermoplasma* by its characteristic lobed shape, regularly structured coat, and hexosamine content (12, 204). The question will be answered conclusively once DNA hybridization studies are carried out on the DNAs of the two organisms. Nevertheless, there can be little doubt that *T. acidophilum* is phylogenetically much closer to the other acidophilic and thermophilic procaryotes, not included in the class *Mollicutes*, than to the parasitic organisms presently included in this class. De Rosa et al. (72) have, in fact, suggested assembling all the acidophilic and thermophilic procaryotes, including *T. acidophilum*, into a "form/habitat" group named *Caldariella*.

NUTRITION AND GROWTH IN CULTURE

Recent research on mycoplasma nutrition concentrated on searching for cultivation media for the insect and plant mycoplasmas and on defining the nutritional requirements of the more "exotic" mycoplasmas, including the ureaplasmas and thermoplasmas. Studies on the nutritional requirements of the classical animal mycoplasmas were limited, and, consequently, little information can be added to that reviewed previously (235, 236).

Serum Components As Nutrients

The main function of the serum component in mycoplasma media is to provide fatty acids and cholesterol in an assimilable, nontoxic form for membrane synthesis (236). The development of standard fractionation techniques for serum components enabled the analysis of their role in mycoplasma growth. Among the three main classes of human serum lipoproteins, namely, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), LDL was most effective in donating cholesterol, and HDL was least effective (295, 296). VLDL, though capable of functioning as a cholesterol donor, inhibited growth of *A. laidlawii* and *M. hominis*, but not of *M. capricolum* (297). The supposition that VLDL was toxic due to large quantities of fatty acids liberated from the VLDL triglycerides by the mycoplasma lipase failed to gain experimental support (297). In bovine and horse sera the greatest growth-promoting activity, with *M. pneumoniae* and *M. arthritidis* as test organisms, was associated with HDL, which is the major cholesterol-carrying lipoprotein in these sera (R. L. Washburn and N. L. Somerson, personal communication). The albumin component of serum functions as a carrier and detoxifier of the fatty acids required for mycoplasma growth (236). However, when lipid-extracted bovine serum albumin was added to the medium at concentrations of 0.8% and higher, growth of *M. pneumoniae* and *M. arthritidis* was inhibited (R. L. Washburn, J. H. Hughes, and N. L. Somerson, personal communication). Spector et al. (308) suggested that bovine serum albumin contains six high-energy and a number of low-energy binding sites for free fatty acids. Extensive lipid extraction of bovine serum albumin may free the high-energy sites, allowing fatty acids in the growth medium to bind so tightly to the albumin that they can no longer function as nutrients.

Nucleic Acid Precursor Requirements

The extensive knowledge on the enzymic pathways for de novo synthesis and interconversions of nucleic acid precursors and on the regulation of these processes in *Escherichia coli* and other eubacteria prompted comparative studies on nutritional requirements for nucleic

acid precursors and synthetic capabilities in mycoplasmas. All the mycoplasmas tested, including *A. laidlawii, M. hominis,* and *M. mycoides* subsp. *mycoides* (208, 236, 294), lack the orotic acid pathway for pyrimidine synthesis and the enzymatic pathways for de novo synthesis of purine bases. Therefore, supplementation of at least one purine and one pyrimidine base is essential for mycoplasma growth. An extensive study relating nucleotide interconversions in *M. mycoides* subsp. *mycoides* to nutritional requirements has been recently reported (208, 294). This mycoplasma can synthesize all the nucleotides it requires for growth from guanine, uracil, and thymine. Purine nucleotide monophosphates are synthesized by phosphoribosyl transfer to the appropriate base. When nucleosides are supplied in the medium, they are first transformed to the corresponding bases by nucleoside phosphorylase and are then used for nucleotide synthesis by the phosphoribosyl transferase. The ability of *M. mycoides* subsp. *mycoides* to use guanine, but not adenine, as a source of cell purine nucleotides is consistent with an irreversible pathway converting guanine to adenine nucleotides. In conclusion, this mycoplasma appears to possess most of the major pathways available for salvage synthesis of nucleotides, as well as the complex mechanisms for regulating synthesis (208, 294).

Pyrimidine and purine phosphorylases, common enzymes in mycoplasmas, carry out a reversible reaction by which purine or pyrimidine nucleosides in the presence of phosphate are transformed to the corresponding free bases plus ribose-1-phosphate (118). These enzymic activities received special attention when they were found to affect the nucleic acid metabolism of cell cultures contaminated by mycoplasmas. The contaminating mycoplasmas transform the nucleosides supplied in the culture medium to the purine or pyrimidine bases, which are very poorly utilized by most eucaryotic cells in culture. This finding led to the development of several biochemical tests for the detection of cell culture contamination with mycoplasmas (286).

Nutrition of Ureaplasmas

The ureaplasmas differ from the classical mycoplasmas in their ability to hydrolyze urea and their failure to grow in conventional mycoplasma media to titers higher than 10^7 colony-forming units per ml (291). Comparing the growth curves of a ureaplasma with that of a classical mycoplasma (Fig. 1) reveals similar lag and early logarithmic phases in both, but the logarithmic phase in the ureaplasma culture ends rather abruptly once the titer reaches 10^6 to 10^7 colony-forming units per ml. The decline

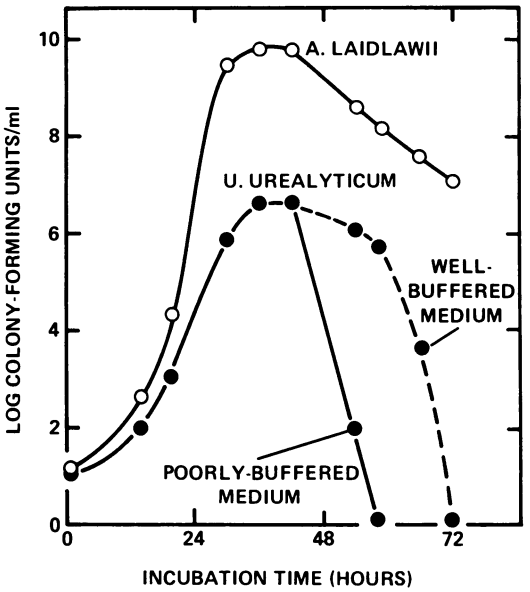


FIG. 1. *Growth curves of Ureaplasma urealyticum compared with that of A. laidlawii.*

phase which follows can be very steep in a poorly buffered medium and less steep when the medium is well buffered (193, 242, 243). As a result of this peculiar behavior, the cell yields of ureaplasma cultures are extremely low. Though, on the basis of the growth curve, one would expect about 1 to 0.1% of the yield of a classical mycoplasma, in practice, a yield of 1 mg of sedimentable protein per liter of ureaplasma culture is quite common (152, 242). Moreover, even this minute pellet consists not only of cell protein but also of a significant amount of proteins which precipitated from the medium and cosedimented with the ureaplasma cells (194). The very small yields of ureaplasma cells severely hamper biochemical and serological studies on these organisms.

Is the restricted in vitro growth of ureaplasmas a result of exhaustion of an essential nutrient from the medium or of the accumulation of a toxic substance? The most likely nutrient to be depleted in ureaplasma cultures is urea. The discovery that urea is required for ureaplasma growth is especially interesting, since no other procaryote or eucaryote shares this requirement (242, 291). The finding of a potent urease in ureaplasmas suggested that growth of these organisms ceases once all the urea in the medium is hydrolyzed, but the failure to improve ureaplasma growth by continuous urea supplementation (193) discredits this explanation. Another explanation can be based on the possible toxicities of CO_2 and ammonia, the products of urea hydrolysis. CO_2, however, not only fails to inhibit

ureaplasma growth but actually improves it due to its buffering capacity at pH 6.0 to 6.5, the optimal pH for ureaplasma growth (243, 291). The second hydrolysis product, ammonia, might inhibit ureaplasma growth. The question is whether the inhibitory effect is directly due to toxicity of the ammonium ion itself or indirect, due to the rise in pH of the medium. Recent data by Masover et al. (193) favor the second possibility by showing that when an acidic pH in the culture medium is maintained by bubbling CO_2, the accumulated NH_4^+ does not increase the death rate. In the absence of an effective buffer, however, the ammonium ions do inhibit growth by increasing the pH of the medium. In fact, the removal of ammonia either by growing the ureaplasmas in very shallow layers under an air flow (121) or by adsorption of the ammonium ions onto an ion-exchange resin added to the growth medium (356) prevented the steep decline in the growth curve, considerably prolonging the stationary phase. Yet, the maximum titer obtained under these conditions never exceeded 10^7 colony-forming units per ml, which suggests that ammonia is not the factor responsible for the low peak titers characteristic of ureaplasma cultures. According to Furness (91), a slowly dialyzable, thermostable, and catalase-resistant substance which accumulates in ureaplasma cultures is responsible for cessation of growth and low cell yields. Clearly, more data are needed to confirm or reject this hypothesis.

The role of urea in ureaplasma growth is still an enigma. Neither CO_2 nor ammonia can replace urea (191, 193), and the radioactivity incorporated by growing ureaplasmas in a medium containing [^{14}C]urea was negligible (82, 242). Though some of the ammonia liberated by urea hydrolysis may be assimilated by the organisms, the fact that it cannot replace urea indicates that supplying assimilable ammonia is not the main role of urea in ureaplasma growth. The possibility that urea may serve as an energy source is attractive, since ureaplasmas were reported to lack the conventional mechanisms for adenosine 5'-triphosphate (ATP) generation in mycoplasmas, such as glycolysis or arginine breakdown (16). Hydrolysis of 1 mol of urea to CO_2 and NH_3 at pH 6.0 releases about 15 kcal (338). However, there is no known pathway in ureaplasmas by which this energy can be trapped and utilized. Attempts to detect enzymes linking the breakdown of urea to the formation of energy-rich carbamoyl phosphate by carbamate kinase have failed (338). A hypothesis based on ion gradient formation through urea hydrolysis has been suggested by Masover et al. (194). This hypothesis stems from the localization of the potent constitutive urease in the ureaplasma cytoplasm (70, 192, 194, 195, 338). At physiological pH, urea is uncharged and presumably permeates the cell membrane freely (194). When hydrolyzed inside the cell, it yields CO_2 and ammonia, which, at physiological pH, accepts a proton, becoming the ammonium ion (NH_4^+). The charged ammonium ions diffuse very slowly across the membrane, producing an ion gradient and a membrane potential. The resulting energized membrane state might be coupled to ATP formation through the membrane-bound adenosine triphosphatase (ATPase) of the ureaplasmas (194). According to this hypothesis, inhibition of urease activity would eventually lead to growth inhibition, and, in fact, the urease inhibitor acetohydroxamic acid (195) and related derivatives of hydroxamic acid did inhibit ureaplasma growth (81, 190). Apart from the lack of experimental data to support the membrane potential hypothesis, it is difficult to reconcile with it the replaceability of urea by putrescine, agmatine, or allantoin (190–192). More recent studies suggest, however, that these amines act synergistically with urea rather than replace it completely (190, 243). Whether or not the amines are transported into the ureaplasma cells and hydrolyzed by an amine oxidase is still unknown, though the low amounts of ammonia produced in ureaplasma cultures grown with putrescine instead of urea (192) do not favor this possibility.

Nutrition of Thermoplasmas

It is not surprising that *T. acidophilum*, being the only known free-living nonparasitic *Mollicute*, has much simpler nutritional requirements than the parasitic mycoplasmas. All the *T. acidophilum* isolates can be grown in a medium consisting of inorganic ions, glucose, and low concentrations of yeast extract (58). The growth-promoting factor supplied by the yeast extract appears to be one or more oligopeptides, which bind avidly to cations (305). Smith et al. (305) propose that the oligopeptide(s) may function as an ion scavenger for some trace metal requirement, protect the organism at its surface from high H^+ concentration, participate in ion transport, or supply essential amino acids in a form able to permeate the cells in the extremely acid environment. The habitat of thermoplasmas, the coal refuse pile, is rich in carbon and organic material. Activated carbon was shown to adsorb and concentrate the essential growth factors found in yeast extract and to release them in the hot and acid medium used to grow *T. acidophilum* (305). Since carbon is probably required to concentrate the oligopeptides needed by the organisms, the natural habitat of thermoplasmas is restricted to hot and carboniferous areas (305).

ENERGY-YIELDING MECHANISMS

The parasitic mycoplasmas have truncated respiratory systems, lacking quinones and cytochromes (236). Of the *Mycoplasma, Acholeplasma, Spiroplasma,* and *Thermoplasma* species examined by Hollander et al. (124), only *T. acidophilum* contained micromolar concentrations of quinones per gram of cell protein, whereas all the others contained only nanomolar concentrations, apparently adsorbed from growth medium components. Micromolar concentrations are necessary for quinone-mediated, energetically useful electron transport in bacteria. Since cytochromes have also been detected in *T. acidophilum* (12, 124), it appears that this free-living mycoplasma has a complete electron transport chain and may thus employ the highly effective oxidative phosphorylation mechanism for ATP generation. In fact, aeration considerably promoted growth of *T. acidophilum* (124), whereas it had no significant effect on growth of parasitic mycoplasmas (184). *M. arthritidis* strain 07 has long been a notable exception to the rule that parasitic mycoplasmas lack a complete electron transport chain. P. J. VanDemark and P. F. Smith showed in the mid-1960s that this strain possesses a complex electron transport chain consisting of flavoproteins, quinones, and cytochromes and that it is capable of oxidative phosphorylation (235, 236). Since in all other respects, including genome size, *M. arthritidis* resembles other parasitic mycoplasmas, such metabolic complexity is somewhat unexpected. The recent findings of Hollander et al. (124) that *M. arthritidis* strain 07 has only minute quantities of quinones and no cytochromes are therefore noteworthy. The reason for the discrepancy between the old and new data is not yet clear. If confirmed, however, the data of Hollander et al. (124) will strengthen the generalization that parasitic mycoplasmas possess a truncated respiratory pathway.

Another indication for the simplicity of the electron transport chain in parasitic mycoplasmas is the finding that in *Mycoplasma* species (226) and in *S. citri* (148, 211) the reduced nicotinamide adenine dinucleotide (NADH) oxidase activity, representing the transfer of electrons from the oxidized substrate to oxygen, is in the cytoplasmic fraction of the cells. That the NADH oxidase is really cytoplasmic and is not released from the membrane during cell fractionation is supported by Z. Ne'eman's finding (Ph.D. thesis, Hebrew University, Jerusalem, Israel, 1974) that the NADH oxidase activity of *M. mycoides* subsp. *mycoides* was not associated with the membrane even when cell lysis was carried out by digitonin in the presence of Mg^{2+}, a condition minimizing the release of loosely associated membrane proteins. Furthermore, histochemical localization of tellurite reduction showed that it takes place in the cytoplasm of *M. mycoides* subsp. *mycoides* (339). Complex electron transport chains are usually membrane bound, since they depend on the spatial organization of their components, as is the case for the NADH oxidase systems in most eubacteria (171, 226). Unlike that in *Mycoplasma* and *Spiroplasma* species, the NADH oxidase activity in *Acholeplasma* species is membrane bound (226). Yet, the *Acholeplasma* system resembles that of the *Mycoplasma* in being composed of only two enzymic proteins associated with flavins (140, 141, 162, 171).

Ruling out oxidative phosphorylation as an ATP-generating system in most, if not all, parasitic mycoplasmas leaves only two proven ways of ATP generation, both based on substrate-level phosphorylation. In fermentative mycoplasmas, ATP is formed during glycolysis, whereas in nonfermentative mycoplasmas, the arginine dihydrolase pathway has been proposed as the major source for ATP (285).

$$\text{arginine} + H_2O \xrightarrow{\text{arginine deiminase}} \text{citrulline} + NH_3$$

$$\text{citrulline} + \text{inorganic orthophosphate} \underset{}{\overset{\text{ornithine carbamoyltransferase}}{\rightleftharpoons}} \text{ornithine} + \text{carbamoyl phosphate}$$

$$\text{carbamoyl phosphate} + \text{adenosine 5'-diphosphate} \underset{Mg^{2+}}{\overset{\text{carbamate kinase}}{\rightleftharpoons}} ATP + CO_2 + NH_3$$

The arginine deiminase is inducible and becomes a major cell protein on induction (80, 285; D. E. Fahrney, personal communication). The purified *M. arthritidis* arginine deiminase has a molecular weight of about 80,000 and probably exists as a dimer (343). The question is whether the ATP produced by this pathway can satisfy all the energy requirements of the cells. Schimke et al. (285) based their positive answer to this question on the marked growth stimulation of the nonfermentative arginine dihydrolase-positive mycoplasmas on adding L-arginine to the medium, a finding which has since been confirmed by others (80, 235). Moreover, Fahrney (personal communication) has found that *M. arthritidis* cells adapted to grow on arginine become completely dependent on it as an energy source, and inhibitors of the arginine deiminase activity suppress mycoplasma growth. Nevertheless, there are some indications that the arginine dihydrolase pathway may not function as

a major energy-generating system, at least in some of the arginine dihydrolase-positive mycoplasmas. Thus, *S. citri,* which belongs to the small group of mycoplasmas having both the glycolytic and the arginine dihydrolase pathways (114), grew very poorly with arginine as the sole energy source (326). Fenske and Kenny (80) argue that if the major energy-generating system in nonfermentative mycoplasmas is the arginine dihydrolase pathway, it should be well regulated so that a good correlation would be found between CO_2 production, cell numbers, and arginine deiminase activity. This was not the case, as CO_2 production catalyzed by carbamate kinase was not affected by the arginine concentration in the growth medium. Moreover, their observation that induction of arginine deiminase activity occurred only in the late logarithmic phase of growth suggests that induction followed the exhaustion of some other energy-yielding substrate.

Another possible mechanism for ATP generation in mycoplasmas has recently been proposed by Kahane et al. (149a). This mechanism is based on ATP generation from acetyl phosphate and adenosine 5'-diphosphate by acetate kinase, coupled with acetyl phosphate formation from acetyl coenzyme A (acetyl-CoA) by phosphate acetyltransferase; both enzymes are commonly found in fermentative and nonfermentative mycoplasmas.

acetyl-CoA + inorganic orthophosphate

$$\xrightleftharpoons{\text{phosphate acetyltransferase}}$$

acetyl phosphate + CoA

acetyl phosphate + adenosine 5'-diphosphate

$$\xrightarrow[3]{\text{acetate kinase}}$$

acetate + ATP

Acetyl-CoA can be produced by oxidative decarboxylation of pyruvate by mycoplasmas. The direction of the reversible acetate kinase reaction favored ATP synthesis with the purified enzyme from *A. laidlawii* (I. Kahane and A. Muhlrad, unpublished data).

Energy coupling through the formation of a membrane potential according to the Mitchell chemiosmotic theory is an attractive possibility for ATP generation in mycoplasmas, but there is little experimental evidence to support this. The proposal that in ureaplasmas the intracellular breakdown of urea and accumulation of NH_4^+ lead to the formation of a membrane potential has been discussed in detail in Nutrition and Growth in Culture. Evidence for the existence of a membrane potential is available for *T. acidophilum.* The intracellular pH in this organism was estimated at between pH 5.5 and 6.9, depending on the technique used for its measurement (130, 273, 288). Since the pH of the growth medium was about 2.0, a pH gradient of 3.5 to 4.9 must exist between the outside and the inside of the cells. The finding that metabolic inhibitors and proton-conducting uncouplers did not affect this gradient led Hsung and Haug (130, 131) to conclude that it is maintained passively by a Donnan potential across the membrane, possibly generated by charged intracellular macromolecules. $KS^{14}CN$, known to penetrate biological membranes, accumulated in the *T. acidophilum* cells, whereas tetraethylammonium bromide, a lipophilic cation, did not, suggesting that the cells are positive on the inside, whereas their surface has a highly negative charge. Hence, some positively charged ions or macromolecules, possibly including the histone-like proteins described by Searcy (287), must be present within the cells to create the Donnan potential (132). At pH 2.0, the measured membrane potential was about 120 mV, positive inside, compensating only partly for the huge pH gradient. The membrane potential decreased linearly on increasing the external pH, diminishing to less than 15 mV at pH 6.0 (131). Hsung and Haug (131) consider whether ATP can be generated in *T. acidophilum* according to the Mitchell chemiosmotic theory. According to their calculations, the net electrochemical gradient in resting cells at 56°C and pH 2.0 is approximately 170 mV (290 mV due to the pH gradient minus 120 mV of the Donnan potential). Though this value is much lower than the 300 mV required for ATP synthesis according to the Mitchell theory, it was calculated from data obtained with resting cells, so that a definite answer to the question is unavailable as yet.

CELL MORPHOLOGY AND REPLICATION

The heated controversies of the 1960s on the "true" morphology, minimal size, and mode of replication of mycoplasmas have calmed down, and concensus has been reached on most points. Current research is focused on some basic problems, including the mechanism of cell constriction preceding cytoplasmic division, the factors responsible for the helical shape of spiroplasmas, and the nature of organelles associated with mycoplasma attachment to host cells.

Minimal Size

The experimental data and theoretical considerations which led to the conclusion that the

smallest mycoplasma cell capable of reproduction is about 0.3 μm in diameter were reviewed previously (182, 209, 236). Recent observations by Robertson et al. (252) support the above conclusion by showing that the small spherical bodies (0.10 to 0.25 μm in diameter) isolated from *M. hominis* cultures by filtration without squeezing could not reproduce.

Filament Formation

As is to be expected from plastic organisms, the coccus is the basic form in all mycoplasma cultures. In most, and under certain conditions perhaps in all, mycoplasma cultures, elongated or filamentous forms can also be observed (18, 213, 236). *M. pneumoniae* filaments attached to epithelial cells were observed in sputum of pneumonia patients (53), indicating that filamentous growth occurs in vivo as well. The excellent film by Bredt et al. (27; film E 1813 of the Encyclopaedia Cinematographica, Gottingen, West Germany) of growing *M. hominis* cultures observed under the phase-contrast microscope is the best reference for studying cell morphology, growth, and replication. The viewer can follow the formation of filaments, measure their elongation rate (reaching 0.5 to 1.7 μm/min), and observe the subsequent transformation of the filaments into chains of cocci by constrictions in the cell membrane (Fig. 2).

How and why is a filament formed? Obviously, surface forces acting on the plastic mycoplasma cell tend to preserve the spherical shape; hence, for the cells to grow as filaments, an axial force must be maintained. Although the exact nature of the axial force is still unknown, I propose that filament formation in mycoplasmas is a function of the growth rate. The effect of growth rate on cell length is well known for wall-covered bacteria; for example, *Arthrobacter* sp. grown in a chemostat produced rods when the growth rate was fast and cocci when the growth rate was slow (172). Thus, it may be suggested that in fast-growing mycoplasmas genome replication precedes cytoplasmic division, resulting in the formation of multinucleate filaments. The absence of filament formation in mycoplasma cultures growing in deficient media (235) is in accord with this hypothesis. Moreover, the rare appearance of filaments in ureaplasma cultures (243) might be the expression of their poor growth in the presently available media. When looking for filaments in mycoplasma cultures, it should also be remembered that the filamentous phase is transitory and relatively short (227, 235), and it can be missed if growth is not followed continuously.

Cell Division

The film on *M. hominis* cultures (27) provides the most vivid and conclusive evidence for the reproductive cycle proposed by Freundt (87), in which filaments formed from coccoid cells are transformed into chains of cocci (Fig. 2) which subsequently fragment into single coccoid cells. The film shows that the transformation of the filament to chains of cocci starts either on one part of the filament, spreading rapidly over its entire length, or simultaneously at several points on the filament. The transformation is rapid and may be completed within 2.5 min.

We have no idea as to the identity of the agent(s) signaling the filament to fragment, but we might be close to understanding the mecha-

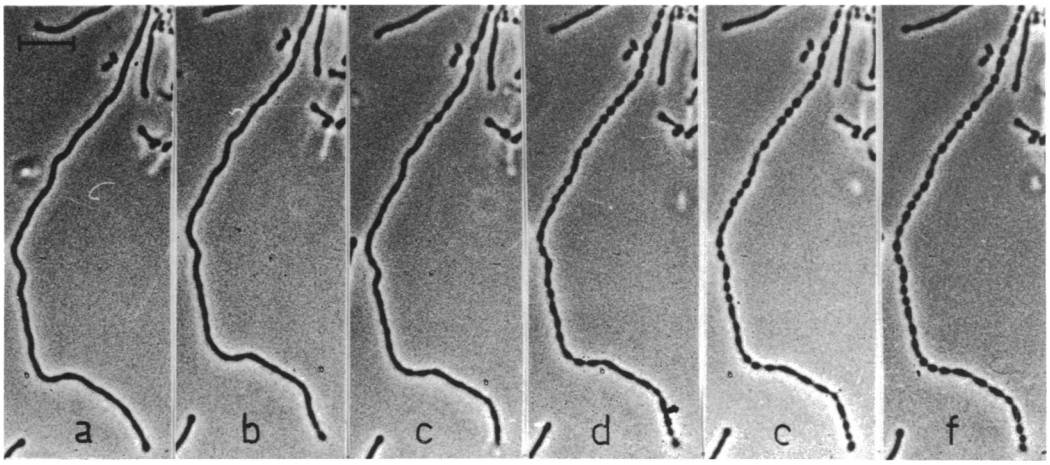


FIG. 2. *Transformation of a filament to a chain of cocci. Cinematographic pictures of an M. hominis culture growing in a cover slip chamber under the phase-contrast microscope. Time intervals of pictures from (a), in min: (b) 2.8; (c) 3.3; (d) 4.8; (e) 5.3; (f) 5.5. From Bredt et al. (27).*

nism responsible for the constriction process preceding cytoplasmic division. The ability of the living mycoplasma cell to contract, expand, and change shape was noted long ago by dark-field microscopy of *M. mycoides* (313) and recently by microcinematography of viable *M. hominis* (27). Apart from demonstrating the highly dynamic nature of the living mycoplasma cell, a factor to be considered in evaluating morphological shapes obtained with fixed nonviable mycoplasmas, these observations strongly indicate the presence of contractile material in mycoplasma cells, and, in fact, evidence for actin-like proteins in mycoplasmas is accumulating (see Motility and Contractility).

The problem of whether reproduction of mycoplasmas can be considered as binary fission was previously discussed at length (182, 235, 236). Recent data have not changed our opinion that the mode of reproduction of mycoplasmas is essentially not different from that of other procaryotes dividing by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronized with genome replication, and in mycoplasmas cytoplasmic division may lag behind genome replication, resulting in the formation of multinucleate filaments.

Helical Filaments

The discovery of the helical shape of plant and insect mycoplasmas (65, 66, 275) raised several interesting questions. Does the life cycle of the helical mycoplasmas resemble that of the classical animal mycoplasmas? Do they reproduce in the same manner? How is the helical shape of the filamentous forms maintained in the apparent absence of axial filaments and cell walls? Phase-contrast or dark-field microscopy of spiroplasma cultures (345) shows that spheroidal bodies predominate in very young cultures and become short, helical filaments at the logarithmic phase. At the late-logarithmic and stationary phases, the helical forms increase in length, at times spectacularly, and aggregates of still-motile entangled filaments appear. In older cultures, helicity and motility are lost, and, as in classical mycoplasma cultures, during the decline phase of growth the filaments disintegrate and irregular coccoid bodies predominate. In a recent study (I.-M. Lee, Ph.D. thesis, University of California, Riverside, 1977), the helical filaments of *S. citri* produced under optimal growth conditions were shown to divide by constriction at several points, producing a number of curved rods or short, helical filaments (Fig. 3). Hence, the morphological changes taking place in developing spiroplasma cultures basically resemble those occurring in mycoplasma cultures, and, in fact, the filaments of *S. citri* can branch as can those of classical mycoplasmas (48, 50, 66, 241). On the other hand, the spiroplasma filaments are much thinner (0.1 to 0.2 μm in diameter) than those of the classical mycoplasmas (0.3 to 0.6 μm in diameter) and usually have one pointed end (332, 354), a feature not observed in classical mycoplasma filaments (Fig. 3). In addition, mostly in older cultures or when grown in a suboptimal environment, the spiroplasma filaments, unlike those of classical mycoplasmas, develop "blebs" (48, 50, 241). These blebs, which might represent bulges of cytoplasm at weakened areas in the cell membrane, are likely to rupture and, by releasing the viscous and sticky cellular DNA, constitute the nucleus of the "medusa"-like aggregates characteristic of the late growth phases of spiroplasma cultures (241).

The most puzzling feature of spiroplasmas is the helicity of their filaments in the apparent absence of any supportive structure. Though this helicity was attributed to a tight packing of

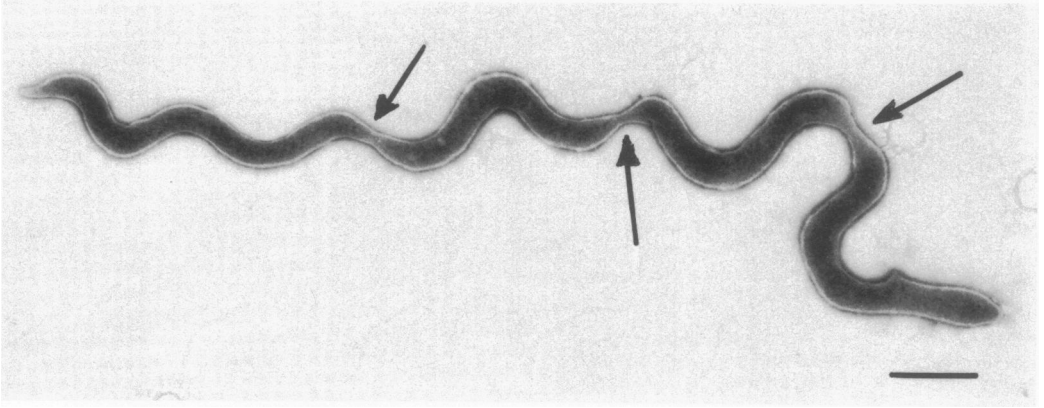


FIG. 3. *Constrictions in the helical filament of S. citri preceding its division. The characteristic shape, of one pointed end and one blunt end, of the filament can also be seen in this negatively stained preparation. Bar = 500 nm. Courtesy of I.-M. Lee.*

the lipid phase of the cell membrane, due to the saturated nature of its lipids (86), this suggestion appears unlikely, since *M. hominis,* with a similar lipid composition to that of *S. citri* (264, 269), is not helical. A plausible hypothesis for spiroplasma helicity can be based on the association of helically wound fibrils with the inner side of the cytoplasmic membrane. When these fibrils are tense or compressed, the cell assumes a helical shape (325). Contraction of these fibrils may also be responsible for the flectional and rotary motion of the spiroplasma helices. Several electron microscopic studies suggest the presence of fibrils in spiroplasmas. Striated fibrils, 3.6 nm in diameter, with a repeat interval of approximately 9 nm along their lengths were observed in several spiroplasmas lysed by deoxycholate (351). However, the possibility that the fibrils are made of aggregated deoxycholate (173) has not been ruled out as yet. Striated structures, 12 nm in width and 25 to 250 nm in length, were seen by Cole et al. (48, 50) in negatively stained flattened *S. citri* cells. These probably correspond to the discontinuous striated structures which in cell sections were seen to adhere to the inner side of the membrane and were probably organized in a helical fashion (Fig. 17c in reference 48). Although it is too early to definitely associate these fibrous structures with helicity, the preliminary studies of H. C. Neimark (Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D62, p. 61), suggesting the presence of actin-like proteins in *S. citri,* support the role of contractile fibrils in forming the helical shape of spiroplasma filaments.

Why is helicity lost upon aging of spiroplasma cultures or during growth under suboptimal conditions, such as acidic pH or inadequate tonicity (48, 50, 86, 241, 345)? *S. citri* is extremely sensitive to osmotic pressure changes and undergoes lysis at even high-speed centrifugation (241). It may be ventured that under suboptimal conditions, when membrane permeability is affected and cells swell, helicity is lost due to the detachment from the membranes of the fibrils associated with helicity. In this context it is interesting to mention that the nonhelical *S. citri* mutant isolated by Townsend et al. (327) still contained fibrils (R. Townsend, personal communication) but lacked a certain membrane protein, so one may speculate that this protein is responsible for the attachment of the fibrils to the cell membrane.

Terminal or Tip Structures

The ultrastructural features seen in thin sections of mycoplasmas have been reviewed previously (182, 236). Ultrastructural analysis of thin sections of the new *Spiroplasma, Thermoplasma,* and *Anaeroplasma* species revealed no features not seen in the classical mycoplasmas, and the same simple ultrastructure, consisting of a cell membrane, ribosomes, and a procaryotic chromosome, has been observed in all the *Mollicutes* examined so far (48, 50, 58, 254, 255). Some advances have been made, however, in the study of the specialized terminal structures appearing in several mycoplasmas and possibly associated with adherence and motility (see Motility and Contractility and Adherence to Cell Surfaces). Both phase-contrast microscopy (26) and scanning electron microscopy (213) showed the filamentous *M. pneumoniae* to have a bulblike "neck" and a tapered tip. In thin sections, the tip was shown to consist of a dense central rodlike core surrounded by a lucent space, enveloped by the cell membrane (355). Since the terminal structures have not been isolated, we have no information on their chemical composition. Indirect evidence obtained with specific stains suggests that the terminal structure of *M. pneumoniae* is composed of basic proteins and is covered by a mucoprotein layer heavier than that on the rest of the cell surface (355). A new *Mycoplasma* species, *M. alvi,* isolated from the alimentary tract of cattle, also possesses a terminal structure resembling that of *M. pneumoniae* (114). Whether this structure is associated with the ability of these parasites to attach to the brush border epithelium of the intestine is still unclear.

MOTILITY AND CONTRACTILITY

The broad phylogenetic diversity among the *Mollicutes* is also reflected in the different modes of motility exhibited by several members of this class. Although some of the classical mycoplasmas "glide" like myxobacteria on liquid-covered surfaces (23, 24), the helical spiroplasmas display a rapid rotary motion and flectional movements (65) resembling those of spirochetes, and *T. acidophilum* is motile at 56°C by means of a long and thin monotrichous flagellum (88; E. A. Freundt, personal communication). The finding of flagellar motion in the wall-less thermoplasma is of special interest, since the transformation of flagellated bacteria into protoplasts or spheroplasts abolishes motility even though the flagella are retained. Thus, it has been suggested that the cell wall fulfills an essential role in bacterial motility, presumably by providing a mechanical support for the rotational flagellar motion (73). The motility of the wall-less *Thermoplasma* by means of flagella obviously poses a serious challenge to the above hypothesis.

Gliding motility on liquid-covered surfaces has been demonstrated in three *Mycoplasma* species only: *M. pulmonis, M. pneumoniae,* and *M. gal-*

lisepticum (23, 24). All three gliding species have specialized structures (a stalk in *M. pulmonis,* a tapered tip in *M. pneumoniae,* and a bleb in *M. gallisepticum* [23, 24, 28, 236]) which serve both as attachment sites to the surface and as the leading parts of the cells during movement. An avirulent *M. pneumoniae* strain which has a reduced ability to adhere to cell surfaces and to glass was found to be immobile (24). The addition of 3 to 5% gelatin to the liquid medium facilitates observation of gliding motility by increasing the viscosity of the medium and improving cell adherence (24). Without gelatin, motility cannot be quantitatively studied, because the moving cells lose contact with the glass and disappear in the liquid medium (232).

Microcinematography showed *M. pulmonis* and *M. pneumoniae* to be much faster than *M. gallisepticum,* reaching speeds of 1 to 2 μm/s (24, 28, 232). The periods of motility were interrupted by resting intervals, which were shortest in *M. pulmonis* and longest in *M. gallisepticum* (28). Although the gliding movement of mycoplasmas is comparable to that of myxobacteria in speed, it differs in that the mycoplasmas move as single cells and not in swarms, and their moving pattern consists mainly of circles and narrow bends. In contrast to myxobacteria, the mycoplasmas never change their leading end (232).

The use of inhibitors to study the mechanism of mycoplasma motility is complicated by the fact that any agent which interferes with attachment of the cells to the surface also inhibits cell motility. Thus, it is not clear whether inhibition of *M. pneumoniae* motility by antibodies to cell surface proteins (232) is caused by inhibition of cell components directly involved in the motility process or is simply the result of interference with mycoplasma attachment (110). The SH-blocking reagents *p*-chloromercuribenzoate and iodoacetate inhibited *M. pneumoniae* motility, whereas cyanide, dinitrophenol, sodium fluoride, and ouabain were ineffective (23). Recent evidence (163) indicates that flagellar motility in bacteria depends on the energized membrane state that can be generated either by oxidation-reduction reactions or by ATPase activity. Flagellar motion is inhibited by uncouplers and by ionophores which cause the collapse of the membrane potential. Therefore, it would be significant to test whether ionophores, such as carbonylcyanide *m*-chlorophenylhydrazone, or the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide affect the gliding motility of mycoplasmas.

Another approach to study the mechanism of mycoplasma motility can be based on nonmotile mutants or variants. *M. pulmonis* usually loses its gliding ability after several subcultures in vitro (23), but so far no study has compared the chemical and physiological properties of the motile and nonmotile cells. The loss of motility in an avirulent *M. pneumoniae* strain which had a reduced capacity to adhere has been mentioned above. The plausible assumption that the nonmotile variant has different cell surface properties has not yet been substantiated. On the contrary, the terminal structure associated with *M. pneumoniae* adherence to surfaces can still be seen in the variant (51), and its cells are able to adsorb the antibodies inhibiting the motility of a motile *M. pneumoniae* strain (232).

Proceeding to spiroplasma motility, Townsend et al. (327) reported the isolation of a nonhelical and nonmotile *S. citri* strain from diseased plants. Electrophoretic analysis of cell proteins showed at least one protein band to be missing or present in very small quantities in the nonmotile strain. This protein may be associated with *S. citri* helicity and motility, which leads us to consider the possibility of the presence of contractile proteins in mycoplasmas. Though procaryotes have been considered to be free of contractile actomyosin-like structures, recent studies indicate that this generalization may not be true. After a report by Minkoff and Damadian (207) suggesting the presence of an actin-like protein in *E. coli,* Neimark (217) provided evidence for the presence of a similar protein in cell extracts of *M. pneumoniae.* This protein resembles eucaryotic actin in solubility in high-ionic-strength salt solutions and insolubility at low ionic strength, in molecular weight, and in its ability to form, in the presence of ATP and Mg^{2+}, long curvilinear filaments 5 to 6 nm wide that closely resemble eucaryotic filamentous actin. It could also bind to vertebrate heavy meromyosin to form hybrid complexes with the characteristic shape of periodic repeating arrowheads. Preliminary data also suggest the presence of actin-like proteins in *M. gallisepticum* (105) and in *A. laidlawii* (I. Kahane and A. Muhlrad, Proc. Annu. Meet. Isr. Soc. Biochem., Isr. J. Med. Sci. **13**:956, 1977). However, the fibrils observed in deoxycholate-treated spiroplasmas by Williamson (351) are clearly different from actin filaments. That *S. citri* does not contain a protein identical with eucaryotic actin has also been indicated by the extremely sensitive technique of bidimensional electrophoresis of labeled cell proteins of *S. citri.* None of the more than 350 cell proteins detected by this technique co-electrophoresed with muscle actin (A. W. Rodwell, personal communication). In addition, the protein band missing from the nonmotile strain of *S. citri* cannot be identified as actin either, since its molecular weight is definitely lower (39,000 versus 45,000 for actin [Townsend,

personal communication]). Likewise, the protein detected by Minkoff and Damadian (207) in extracts of *E. coli* may be identical with the elongation factor Tu, functioning in the transfer of aminoacyl transfer RNA (tRNA) to ribosomes (258). The elongation factor differs from the actin-like proteins of eucaryotic cells, though it shares many properties with them. Cytochalasin B, an inhibitor of contractile elements in eucaryotes, failed to inhibit the gliding motilities of *M. pneumoniae* (23, 24) and of wall-covered gliding bacteria (122), but it inhibited cell division in *M. gallisepticum* (105). Although actin is one of the few proteins well preserved during the evolution of the eucaryotes (217), it is not likely that an identical protein would be present in procaryotes. Nevertheless, in spite of the many questions still left, the preliminary evidence available does seem to suggest that contractile proteins exist in mycoplasmas. It is hoped that the intensive investigations currently under way will soon clear up the picture.

In all probability unrelated to motility and contractility, but superficially resembling structured fibers of contractile proteins, are the *rho* fibers found in *M. mycoides* subsp. *mycoides* strains grown under certain conditions (222, 256). The *rho* fibers or rods are composed of parallel fibrils about 3 nm in diameter aligned along the long axis of the fiber with structural features in lateral register, producing a cross-banded appearance. The resultant pattern consists of alternate major light and dark bands, the latter bisected by a central minor light band. The pattern has a repeated periodicity of from 12 to 14.5 nm. The rod, which extends throughout the entire organism up to a length of 5 μm, apparently confers a degree of rigidity on the elongated cell (222). The rapid dissolution of the *rho* fibers in low-ionic-strength solutions hampered their isolation, so that to purify them a rather complicated procedure had to be devised (256). The ability of the solubilized fiber material to reassemble spontaneously has been of great value in fiber purification. The reconstituted fibers were found to be built of one protein only (molecular weight, 26,000). Another protein (molecular weight, 85,000) found in the crude fiber preparation is probably involved in the association of the *rho* fibers with the cell membrane, since it could be recovered in the membrane fraction obtained by osmotic lysis of the cells. The functional significance of the *rho* fibers is presently unknown. Since these mycoplasmas are nonmotile, it could not be associated with motility (222). It is possible that the *rho* fiber has no function, but is a paracrystalline aggregate of a fibrous protein synthesized in excess (256).

GENOME

Structure, Size, and Base Composition

The mycoplasma genome is typically procaryotic in consisting of a circular double-stranded DNA molecule (182, 209, 236), but it differs from the genomes of other procaryotes in its small size and low guanine plus cytosine (G+C) content (Table 1). Based on genome size, the mycoplasmas form two clusters: the *Mycoplasma* and *Ureaplasma* species, with a genome size of about 5×10^8 daltons, and the *Acholeplasma, Spiroplasma,* and *Thermoplasma* species, with a genome size of about 1×10^9 daltons (Table 1). As the smallest known genome size of wall-covered bacteria is around 1×10^9 daltons, the *Mycoplasma* and *Ureaplasma* species have the smallest genome recorded for any self-reproducing procaryote (8, 209).

The data on genome sizes of procaryotes reveal a gap between 6×10^8 and 1×10^9 daltons, which, according to Morowitz and Wallace (209), argues against the hypothesis that mycoplasmas developed from wall-covered bacteria by deletion of genetic material. If this were so, one would expect the genome sizes to span all values, from the lowest bacterial value of 1×10^9 to the minimal value of 5×10^8 represented by the *Mycoplasma* genome. Moreover, Walker (340) questions how mycoplasma species with a DNA of such extremely low G+C content could have evolved from any wall-covered eubacteria by deletion of "nonessential" functions, when the genome size of mycoplasmas is still 25 to 50% of that of most eubacteria. Morowitz and Wallace (209), therefore, propose that the existing *Mycoplasma* and *Ureaplasma* species represent the descendants of the organisms which preceded the procaryotic-eucaryotic cell split and suggest designating them "protocaryotes." According to this hypothesis, evolution occurred through DNA doubling, which led to the 1×10^9 daltons of the *Acholeplasma* genome, and, thus, the acholeplasmas can be regarded as intermediates in the evolution from the protocaryotes to the wall-covered procaryotes. The eucaryotic cell is, accordingly, no longer considered as having been derived from the procaryotic cell; rather, both cells developed from the same protocaryotes. Although it is too early to conclude whether mycoplasmas evolved from wall-covered bacteria or vice versa, the genetic evidence discussed above, as well as investigators' failure to show genetic relatedness between mycoplasmas and eubacteria by DNA hybridization techniques (235), speaks against the idea that mycoplasmas represent stable L-forms of existing wall-covered procaryotes.

Analyses of genome size, base composition,

and nucleotide sequence have become indispensable tools in modern bacterial classification. The contribution of these techniques to mycoplasma classification has been particularly impressive because of the scarcity of biochemical assays as well as frequent inadequacy of serological tests (235). One of the major problems plaguing bacterial taxonomists is the classification of bacteria into species, subspecies, and varieties, since the definition of species in bacteria is vague. The advantage of using DNA analysis for classification purposes can be illustrated through a report by Askaa and Ernø (9) dealing with the question of whether the two subspecies of *M. agalactiae* (subsp. *agalactiae* and subsp. *bovis*) should be separated into two different species. Biochemical and serological tests failed to clearly answer this problem. DNA hybridization tests, however, showed only 38 to 40% homology between the DNAs of the two subspecies (9). Since the degree of relatedness at the subspecies level in bacteria, as proposed by Johnson (144), is 60 to 70% homology, it was decided to separate the two subspecies of *M. agalactiae* into two species: *M. agalactiae* and *M. bovis* (9). Clearly, an absolute criterion, such as percent DNA hybridized, simplifies classification, but it must be stressed that the limit of 70 to 80% homology set for subspecies relatedness is arbitrary; in nature we may frequently encounter a continuous range of DNA homology values among related strains. In fact, though the 40% DNA homology was judged insufficient to classify the *M. agalactiae* subspecies together, it can still be regarded as expressing a significant degree of genetic relatedness, as reflected by similar biochemical reactions and electrophoretic patterns of cell proteins, and some degree of serological relatedness (9). A promising approach to mycoplasma classification, recently introduced by Bové et al. (J. M. Bové, personal communication), is based on the hydrolysis of mycoplasmal DNA by a series of restriction enzymes. The resulting oligonucleotides exhibited highly reproducible and strain-specific patterns on electrophoresis in polyacrylamide gels. The small genome size of mycoplasmas is advantageous, for when the much larger genome of *E. coli* was digested with the restriction enzymes, the number of different oligonucleotides produced was too great to be properly separated in the gels.

Studies on the *T. acidophilum* genome are important because of the questionable relationship between this wall-less, free-living procaryote and the parasitic mycoplasmas. The early report that the DNA of *T. acidophilum* resembles that of the parasitic mycoplasmas in having a G+C content as low as 25% (12, 58) proved to be incorrect. The actual G+C value is about 46% (42, 289), higher than in any of the known parasitic mycoplasmas. The genome size of *T. acidophilum*, on the other hand, was found to be about 10^9 daltons (42, 289), closely resembling that of the parasitic *Acholeplasma* and *Spiroplasma* species and representing the smallest genome recorded for any nonparasitic procaryote. Another interesting feature of the *T. acidophilum* genome is its association with a histone-like protein (287). Histones, basic proteins associated with the nuclear DNA of eucaryotes, have not been found in procaryotes. The histone-like protein of *T. acidophilum* resembles eucaryotic histones in having a high basic amino acid content, but it differs in being unusually rich in amides of acidic amino acids. Recent data (D. G. Searcy, D. B. Stein, and G. R. Green, BioSystems, in press) suggest that the histone-like protein condenses the DNA into subunits that are 5 to 6 nm in diameter, each consisting of approximately 40 base pairs of DNA looped around 4 or 6 of protein molecules. Thus, the nucleoprotein of *T. acidophilum* has a subunit structure similar to that of eucaryotic chromatin but of a simpler nature, as the eucaryotic subunits are larger and contain 8 histone molecules plus 130 to 140 base pairs of DNA each. The association of the histone-like protein with *T. acidophilum* DNA acts to stabilize it against thermal denaturation (D.B. Stein and D.G. Searcy, personal communication). Furthermore, this association may also protect the DNA from depurination in the hot and highly acidic environment of *T. acidophilum*. Even though thermoplasmas are capable of maintaining a relatively high intracellular pH, estimated at 5.6 (288) or 6.4 to 6.9 (130), the rate of depurination of unprotected DNA at these pH values may be high enough to impose a severe mutational load (D.G. Searcy, personal communication). Searcy (287) suggests that histones may have evolved independently in eucaryotes and *T. acidophilum*. However, it is also possible that an organism related to *T. acidophilum* was the ancestor of eucaryotic cells, and, in this case, histones may have first evolved to protect its DNA from thermal denaturation or depurination. Once evolved, the histones condensed the DNA and thus were a preadaptation for the accumulation of more DNA in the eucaryotic cells. The similar properties of the *T. acidophilum* histone and histones of primitive eucaryotes, such as *Neurospora* and dinoflagellates, are consistent with this hypothesis (287).

Genome Replication

The replication of the mycoplasma genome resembles that of other procaryotes in being

semiconservative and in proceeding sequentially from, at most, a few growing points (298). By synchronizing *M. gallisepticum* growth, Quinlan and Maniloff (231) showed that DNA is synthesized continuously during cell growth, but not during the phase of cell division. This gap in DNA synthesis occurs in other procaryotes and eucaryotes and represents the time required for new initiator molecules, or some membrane component, to be produced, enabling the reinitiation of chromosome replication. Early evidence that the DNA growing point in mycoplasmas is membrane associated has been summarized by Maniloff and Morowitz (182) and by Razin (236). More recent work (183) suggests that in *M. gallisepticum* the membrane-associated DNA replication complex is located in the peculiar bleb-infrableb terminal structure. A subcellular fraction isolated from cells lysed by alternate freezing and thawing and brief sonic treatment, consisting of membrane vesicles with attached bleb-infrableb structures, contained only 9% of the total cell DNA but 64% of the nascent DNA. This subcellular fraction also contained a unique DNA region permanently associated with the membrane, probably representing the chromosome origin (231). The appearance of the bleb-infrableb structures at the opposite poles of *M. gallisepticum* cells before cell division, in addition to cytological evidence suggesting the synthesis of new bleb-infrablebs at preexisting regions, led Maniloff (178) to propose that the bleb-infrableb structures be regarded as the procaryotic analogs of the eucaryotic centriole. Although this analogy may hold for *M. gallisepticum,* it clearly does not apply to any mycoplasma lacking terminal structures. It may emphasize the uniqueness of *M. gallisepticum,* which, according to Maniloff (178), is the mycoplasma furthest removed from the eubacteria.

The need for DNA polymerase in the replication and repair of the mycoplasma DNA is self-evident, but data on this enzyme system were unavailable until recently. Miller and Rapp (203) detected in cell cultures contaminated with *M. hyorhinis* a DNA polymerase sedimenting at 6S, in addition to the 8S DNA polymerase of the cultured cells. More recently, Mills et al. (205) characterized the DNA polymerases of *M. hyorhinis* and *M. orale,* and, since they were essentially identical in their physical and catalytic properties, they may be prototypic of the mycoplasmal DNA polymerases. The polymerase had a sedimentation coefficient of 5.6S in high salt and an apparent molecular weight of 130,000, a value within the range reported for *E. coli* DNA polymerases I, II, and III. Mills et al. (205) obtained no evidence for the existence of more than a single DNA polymerase species in either of the two mycoplasmas tested, a finding which is in accord with the notion that mycoplasmas economize on genetic information; however, they note that polymerases II and III were found in *E. coli* only after the isolation of *polA* mutants, so it is premature to conclude that mycoplasmas contain a single DNA polymerase. The strong preference of the mycoplasma enzymes for gapped ("activated") DNA suggests that they resemble bacterial DNA polymerases II and III in carrying out a repair type of gap-filling synthesis but differ from them in their ionic-strength response and moderate sensitivity to sulfhydryl-blocking reagents. The purified mycoplasma polymerase was essentially free of endonuclease and thymine dimer-excising activities. Most surprising was the complete absence of associated exonuclease activities, particularly the 3′,5′-exonuclease function, from the most highly purified mycoplasma enzyme preparations; exonuclease activity has invariably been found in purified eubacterial DNA polymerases, with perhaps the single exception of *Bacillus subtilis* DNA polymerase II. In this respect, the mycoplasma enzymes resemble eucaryotic DNA polymerases (205).

Irradiation Damage and Repair

Ultraviolet or X- and gamma-ray irradiation of *A. laidlawii* cultures usually yields survival curves with an initial shoulder followed by exponential inactivation (182). A curve of this shape, characteristic of multihit or multitarget kinetics, may be due to cell clumping or polyploidy, both common features of mycoplasmas in culture. However, the shape of the curve may also be indicative of repair of the DNA damaged by the irradiation. Cellular DNA undergoes degradation after irradiation (74, 182). The suggestion of Drásil et al. (74) that the exonuclease activity of the mycoplasma DNA polymerase may be responsible for DNA degradation is not supported by the recent finding that mycoplasmal DNA polymerase lacks exonuclease activity (205). Degradation may, however, be caused by an endonuclease activated by the irradiation. Oxygen removal, or the addition of sulfhydryl-containing compounds, decreased irradiation damage markedly (37), suggesting that peroxidation of membrane lipids may contribute to irradiation damage. The low superoxide dismutase activity in *A. laidlawii* (223) is apparently insufficient to protect the cells from the peroxides produced during irradiation in the presence of oxygen.

Repair of DNA damage caused by ultraviolet irradiation has been demonstrated in *A. laidlawii* only. Both photoreactivation and dark (excision) repair mechanisms were demonstrated in

this organism (62, 182). Particularly interesting, therefore, is the recent report of Ghosh et al. (104) claiming that *M. gallisepticum* has neither dark repair nor photoreactivation mechanisms, so that the induced pyrimidine dimers in the irradiated cell DNA cannot be excised and further DNA replication is blocked. The ultraviolet inactivation curve of *M. gallisepticum* lacks the shoulder characterizing the inactivation curve of *A. laidlawii*. No other procaryote has been reported to lack both kinds of repair system. The finding of Ghosh et al. (104) is both unexpected and surprising, because the ability of an organism to repair DNA damage is essential for maintaining the structural integrity and functioning of its genome under adverse environmental conditions. *M. gallisepticum*, being a respiratory pathogen, is likely to be exposed to irradiation during its transmission from one host to another. Maniloff (178) suggests that, due to the lack of DNA repair mechanisms, *M. gallisepticum* may accumulate base changes, arising from normal environmental perturbations, far more frequently than do other procaryotes. Yet, *M. gallisepticum* is one of the more homogeneous *Mycoplasma* species, with only slight variations in cell protein composition (234) and antigenic properties (164).

Mycoplasma Genetics

Despite the significant developments in research on mycoplasma viruses (see Mycoplasma Viruses) and the potential that the viruses would provide new tools for the study of mycoplasma genetics (182), little progress has yet been made. As discussed in detail previously (182, 236), genetic research in mycoplasmas has been hampered by difficulties in obtaining stable mycoplasma mutants as well as by the unsuccessful application of classical techniques of microbial genetics, i.e., recombination, transduction, transformation, and conjugation. The reasons for failure are unclear. The recent findings of Ghosh et al. (104) that *M. gallisepticum* has no repair system may be linked to the failure of demonstrating recombination in this mycoplasma, since this process requires postreplicative repair. Clearly, this explanation cannot be applied to *A. laidlawii*, in which repair mechanisms exist (104). Although DNA-mediated transformation in mycoplasmas has not been achieved, DNA isolated from the mycoplasma virus MV-L1 was found to transfect not only its natural host, *A. laidlawii*, but also *M. gallisepticum*, which cannot be infected by the whole virus (168). Fragmented bacteriophage genomes give rise to productive infection by recombination. The existence of transfection in mycoplasmas suggests that this would be a useful system for detecting recombinant events in mycoplasmas. Mutants, such as temperature-sensitive mutants of the virus MV-L51 induced by nitrous acid (J. Maniloff, personal communication; A. Liss, personal communication), may provide the tools for using transfection in developing mycoplasma genetics.

Another underdeveloped area of research concerns mycoplasma plasmids. Satellite DNA, revealed by either electron microscopy or density gradient centrifugation or both, in *M. arthritidis*, *A. laidlawii*, and *M. hominis* (182, 360) probably represents plasmids, but nothing is known of their biological properties. The data indicate that the plasmid DNA may be about 20 $\times 10^6$ daltons and that in *A. laidlawii* it may consist of about 35% of the total cellular DNA, suggesting 50 to 100 plasmids per cell (182). Further studies on the nature and function of mycoplasma plasmids and their relationship to the carrier state of mycoplasma virus DNAs are obviously needed.

RIBOSOMAL AND TRANSFER RIBONUCLEIC ACIDS

Mycoplasma ribosomes resemble those of other procaryotes in having a sedimentation coefficient of about 70S, an RNA-protein ratio of about 60:40, and three ribosomal RNA species, 22S, 16S, and 5S (182, 236). RNAs are highly conserved molecules evolutionarily, as expressed both in molecular size and in base composition. Thus, the G+C content of rRNA in wall-covered bacteria only varies from 50 to 54%, whereas the G+C content of the total genomes varies from 38 to 72% (250). Mycoplasma rRNA's, however, differ in some detail from the rRNA's of other procaryotes, as indicated by their lower G+C content (43 to 48%), which is well below that of bacterial rRNA's (182). In addition, the rRNA species in mycoplasmas corresponding to the *E. coli* 23S rRNA sedimented slightly slower in sucrose density gradients, giving an S value of 22 (182).

The recent application by Reff et al. (250) of polyacrylamide-gel electrophoresis to mycoplasma rRNA's has resulted in a better understanding of the relationship between the rRNA's of mycoplasmas and those of other procaryotes. Electrophoresis under nondenaturing conditions, which at least partially preserved the conformation of the rRNA molecules, revealed that the 16S and 22S rRNA's from *Mycoplasma* and *Acholeplasma* species migrate in patterns different from each other and from those of other bacteria. Under the same nondenaturing conditions, the rRNA's from a stable L-phase of *Streptococcus faecalis* comigrated with the rRNA's extracted from its parent bacterium. When ex-

amined under denaturing conditions, using formamide as the denaturing agent, the 23S rRNA's of all the organisms examined comigrated, but the 16S RNAs of the *Mycoplasma* and *Acholeplasma* species migrated somewhat faster than the corresponding 16S RNAs of the eubacteria. A 2.3% difference in molecular weight was calculated, suggesting that the 16S rRNA of *Mycoplasma* and *Acholeplasma* is shorter by about 37 nucleotides than the corresponding rRNA from eubacteria. From these differences in size and conformation of rRNA's, Reff et al. (250) suggest that a significant evolutionary gap exists between the mycoplasmas and other procaryotes, supporting the present classification of the mycoplasmas in a class of their own. Another approach, based on sequencing the rRNA molecules, may yield even more meaningful results than does rRNA size determination. The unpublished data of C. R. Woese and J. Maniloff cited by Ghosh et al. (104) suggest that the 16S rRNA of *M. gallisepticum* lacks some of the oligonucleotide sequences characteristic of eubacteria.

tRNA's, like rRNA's, are highly conserved molecules with respect to size, composition, and function. The conservation of primary structure and base composition of tRNA's and aminoacyl-tRNA synthetases is, in fact, so pronounced that a synthetase from *E. coli* may charge *E. coli*, yeast, and rat liver tRNA's to equal levels (334). The question of whether the small genome size and low G+C content of mycoplasmal DNA has affected the number of tRNA's and their base composition has been investigated recently. The data obtained indicate that the low G+C content of the mycoplasma genome is not reflected in the G+C content of the mycoplasma tRNA's. *M. capricolum* (kid), with a 25% G+C DNA, has tRNA of 53.8% G+C, only 4.4% less G+C than in *E. coli*, and with *A. laidlawii* tRNA the difference was a mere 2% (236). Moreover, the enzymically formylatable methionine tRNA ($tRNA_f^{Met}$) from *M. mycoides* subsp. *capri*, a mycoplasma with a 24% G+C genome, has the highest G+C content recorded for any tRNA, with only two adenosine-uridine base pairs in the stem regions (340). Hence, it is perhaps not surprising that the high-G+C regions in *M. capricolum* DNA, complementary to both tRNA and rRNA, were found by J. L. Ryan and H. J. Morowitz (182) to constitute only 1.4% of the genome, barely enough to code for 44 different tRNA species and the three rRNA species. Hence, one might expect fewer isoaccepting tRNA species, i.e., less redundancy, in mycoplasmas. In fact only one species each of $tRNA_f^{Met}$, enzymically methylatable $tRNA^{Met}$, glycine tRNA, lysine tRNA, and valine tRNA was detected in *M. mycoides* subsp. *capri* (340), and one phenylalanine tRNA ($tRNA^{Phe}$) was detected in *M. capricolum* (156). In *A. laidlawii*, however, two or three isoaccepting tRNA species were detected for several amino acids (236), possibly reflecting the larger genome in this mycoplasma.

The recent elucidation of the complete nucleotide sequences of $tRNA^{Phe}$ from *M. capricolum* (156) and $tRNA_f^{Met}$ from *M. mycoides* subsp. *capri* (341, 341a) supports the highly conserved nature of these molecules by showing the secondary structure of the nucleotide sequence to be arranged in the familiar cloverleaf model. The *M. capricolum* $tRNA^{Phe}$ lacks ribothymidine, but otherwise it has the usual structure with a sequence of twenty bases, including the dihydrouridine stem and loop, identical with the *E. coli* $tRNA^{Phe}$ (156). The main difference, however, between the mycoplasma t RNA^{Phe} and the *E. coli* $tRNA^{Phe}$ is the number and kinds of modified nucleosides with the *E. coli* $tRNA^{Phe}$ having 10 modified nucleosides and the *M. capricolum* $tRNA^{Phe}$ having only 5. Among cellular RNA species, tRNA is unique in containing numerous modified nucleosides, most still having no clear function. On the whole, one might expect that the less genetic information an organism has, the less the variety and the amount of its modified nucleosides, since the enzymes required for their synthesis might be missing, and in fact the tRNA's of *A. laidlawii*, *M. gallisepticum*, *M. hominis*, *M. mycoides* subsp. *capri*, and *M. capricolum* contained fewer modified nucleosides per tRNA molecule than the tRNA's of other organisms (236, 340). Furthermore, the absence of certain modified nucleosides from mycoplasmal tRNA argues against their being essential for the proper functioning of the tRNA. Thus, the isoleucine tRNA from *M. capricolum*, which lacks ribothymidine, could be charged with isoleucine by *E. coli* aminoacyl-tRNA synthetase and mediate polyisoleucine formation in a cell-free system from *E. coli*. Ribothymidine, therefore, is not required for the recognition of tRNA by aminoacyl-tRNA synthetase, nor does it appear to be required for the binding of tRNA to ribosomes (236). Similarly, the $tRNA^{Phe}$ of *M. capricolum* lacks isopentenyladenosine, a modified nucleoside present in the $tRNA^{Phe}$ of almost all the organisms investigated so far; yet, this mycoplasmal tRNA was fully active in promoting phenylalanine incorporation in a cell-free amino acid-incorporating system from *E. coli*, even in direct competition with the homologous *E. coli* $tRNA^{Phe}$. The inevitable conclusion is that the presence of isopentenyladenosine in $tRNA^{Phe}$ is not an absolute requirement for the functioning of this macromolecule (155).

Despite the highly conserved nature of

tRNA's, those originating in procaryotes differ in some properties from those in eucaryotes. As might be expected, the mycoplasmal tRNA's resemble those of the procaryotes, and, thus, the first nucleotide at the 5′ end of $tRNA^{Met}$ of *M. mycoides* subsp. *capri* cannot form a Watson-Crick type of base pair with the fifth nucleotide from the 3′ end, a unique characteristic of procaryotic initiator tRNA's (341). Moreover, the nucleotide sequence of the *M. capricolum* $tRNA^{Phe}$ is closer to the sequence of the *E. coli* $tRNA^{Phe}$ than to that of the corresponding eucaryotic tRNA (156), and extracts of mycoplasmas contained methionyl-tRNA formylase, an enzyme found so far only in procaryotes or in procaryote-like eucaryotic organelles (236, 340). The fact that most aminoacyl-tRNA synthetases from *E. coli* charge the corresponding tRNA's from mycoplasmas (340) also seems to support the close similarity of the mycoplasmal tRNA's to those of other procaryotes. When the $tRNA_f^{Met}$ from *M. mycoides* subsp. *capri* is compared with the corresponding tRNA from procaryotes, it differs in 12 places (not including modifications) from that of *E. coli* $tRNA_f^{Met}$, in 11 places from that of *Thermus thermophilus,* and in only 6 from that of *B. subtilis* (340). It is also interesting to note that the sequence of the *M. capricolum* $tRNA^{Phe}$ (156) is much more similar to the *Bacillus stearothermophilus* $tRNA^{Phe}$ than to that of *E. coli,* suggesting that, in tRNA structure, the mycoplasmas may be closer to the gram-positive than to the gram-negative bacteria (340).

To conclude, the tRNA's isolated and characterized from mycoplasmas resemble the corresponding tRNA's from other procaryotes. The mycoplasmas, however, appear to contain fewer isoaccepting tRNA species and less modified nucleosides, probably reflecting the limitation on biosynthetic ability imposed by the small size and low G+C content of the mycoplasma genome.

CELL MEMBRANE

The mycoplasmas, lacking a cell wall and intracytoplasmic membranes, have only one type of membrane, the plasma membrane. The ease by which this membrane can be isolated and the fact that controlled alterations in its composition are introducible have made mycoplasma membranes most effective and popular tools in biomembrane research. Since an extensive review on mycoplasma membranes was published in 1975 (240), the following discussion will be limited to recent advances and new topics not covered previously.

Membrane Isolation

Techniques for isolation of mycoplasma membranes and criteria for checking the purity of membrane preparations have been described and evaluated in detail by Razin and Rottem (245). Though osmotic lysis is the preferred technique for isolating mycoplasma membranes, lysis by digitonin is gaining popularity, particularly for mycoplasmas which are relatively resistant to osmotic lysis (2, 7, 195). High pH (9.2 to 10.5) has also been applied to induce lysis of the osmotically resistant *M. gallisepticum* (108) and *T. acidophilum* (273). This technique may suffer from the deficiency that some membrane proteins, or even the membranes themselves, may be solubilized at pH values higher than 10.0 (273; S. Razin, unpublished data).

The isolated mycoplasma membranes resemble plasma membranes of other procaryotes in gross chemical composition, being composed mainly of proteins and lipids. The protein comprises roughly two-thirds of the mass of the membrane, the balance being mostly lipid. Detailed information on membrane composition can be found in previous reviews (240, 245). Contamination of mycoplasma membrane preparations with precipitated components of the growth medium may seriously hamper their chemical, enzymic, and antigenic characterization. This problem is usually encountered in either of two cases: in the fast-growing, acid-producing mycoplasmas (such as *M. mycoides* subsp. *capri*), when allowed to grow long enough to decrease the pH of the culture medium to less than 6.0, or with mycoplasmas (such as the ureaplasmas) which grow very poorly in the available culture media. In the first case, the low pH causes precipitation of proteins and lipoproteins from the serum component of the growth medium (22, 38, 359), which cosediment with the cells during harvesting and washing and may consequently cosediment with (359) or even adsorb to the isolated membranes (265). In the case of the ureaplasmas, cell yield is so low (see Nutrition and Growth in Culture) that most of the pellet obtained by centrifugation of the culture consists of noncellular components which will contaminate the isolated membrane preparations. Thus, Masover et al. (195) reported that the protein content of the "membrane" fraction isolated by digitonin treatment of *Ureaplasma urealyticum* growth in unfiltered Hayflick medium was about 25 times higher than the amount of protein in the soluble cytoplasmic fraction. Reduction of the serum content and prefiltration of the growth medium reduced the "membrane"-to-cytoplasmic protein ratio to about 10 (194), a value still much higher than the ratios of 0.5 to

1.0 reported for similar fractions from classical mycoplasmas. Clearly, at least for ureaplasmas grown in vitro, cell-associated exogenous materials should be accounted for in biological and biochemical tests. Some recently reported analyses of ureaplasma membrane preparations (347, 348) are, therefore, questionable, in particular as none of the criteria for checking membrane purity (240, 245) have been fulfilled.

Capsules

Polymeric substances produced and excreted by the cells in the form of a capsule may be lost during membrane isolation. *M. mycoides* subsp. *mycoides* was the first mycoplasma shown to be covered by a capsule made of galactan (236, 240), a polymer possessing toxic properties (see Factors Involved in Pathogenicity). The "fuzziness" on the surface of a sectioned *A. laidlawii* cell may be associated with a hexosamine polymer consisting of *N*-acetylgalactosamine and *N*-acetylglucosamine (322). Of the *Acholeplasma* species, only *A. laidlawii* is capable of producing this polymer (303), but even in this species its quantity may vary significantly, depending on the strain and on growth conditions (I. Kahane, personal communication). The hexosamine polymer appears to be tightly bound to the membrane, as even after prolonged washing a considerable part of it remains associated with the membrane. The claim by Terry and Zupnik (322) that the polymer can be released from the membrane by brief ultrasonic treatment could not be confirmed (J. M. Gilliam and I. Kahane, personal communication). Evidence for capsules in other mycoplasmas is supported solely by electron microscopy. Surface projections and fuzzy layers were demonstrated on the cell surfaces of *M. pulmonis* and *M. gallisepticum* (236), *M. hyopneumoniae* (125), *U. urealyticum* (17), and *S. citri* (48). Capsules staining with ruthenium red were shown in *M. meleagridis* (116), *M. dispar* (253), *M. pneumoniae* (355) and *U. urealyticum* (127; Fig. 4). In none of these cases has the chemical nature of the capsular material been determined. Ruthenium red reacts with a variety of polyanions and has been used to demonstrate the polysaccharide glycocalyxes of eu-

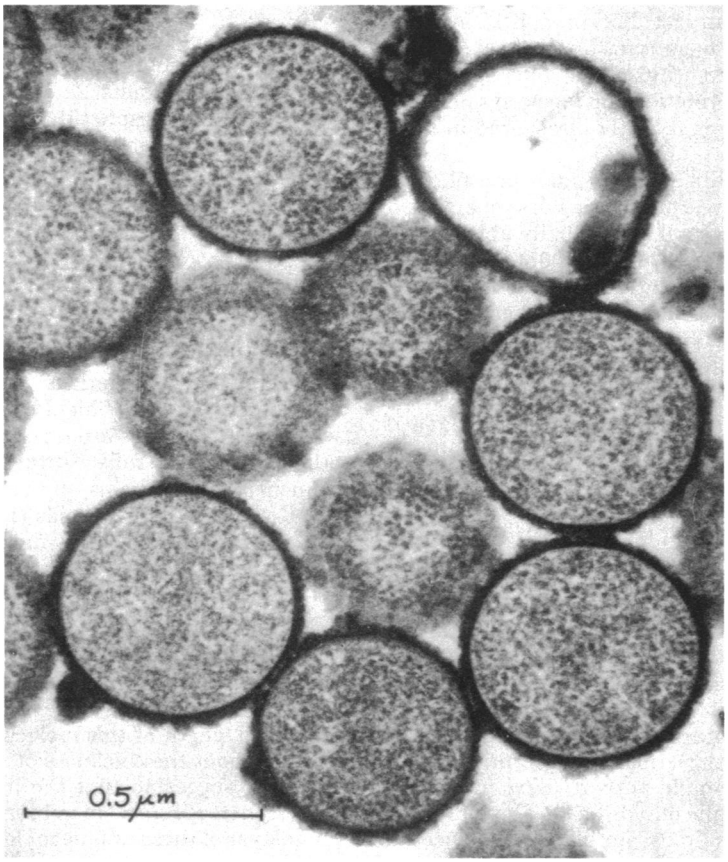


FIG. 4. *Extracellular layer in thin sections of U. urealyticum cells stained by ruthenium red. From Robertson and Smook (253).*

caryotic cells and capsules of procaryotic organisms, including the galactan capsule of *M. mycoides* subsp. *mycoides* (127). Hence, it is probable that in mycoplasmas, too, the extracellular material consists of or contains carbohydrate. In fact, the *U. urealyticum* capsule reacted with concanavalin A-iron dextran, indicating the presence of glucosyl residues in it (253). The possibility that the extracellular material consists of growth medium components adsorbed to the cell surface (253, 282) cannot be ruled out, but it does seem unlikely, as various *Mycoplasma* species (127) and *U. urealyticum* strains (J. Robertson, personal communication) differ in their ability to produce a ruthenium red-positive capsule when grown under the same conditions.

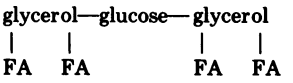
MEMBRANE LIPIDS

Lipid Composition

Virtually all mycoplasma lipids are located in the cell membrane and, as in other biomembranes, consist mostly of phospholipids, glycolipids, and neutral lipids. A detailed description of the composition, distribution, and biosynthesis of mycoplasma membrane lipids can be found in previous reviews (236, 301). This review will update this information and focus on some novel and unique lipids recently discovered in mycoplasmas.

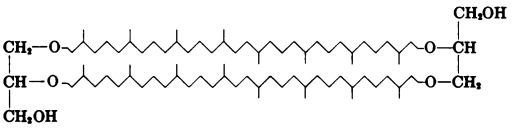
Phospholipids. The acidic phospholipids phosphatidylglycerol and to a lesser extent diphosphatidylglycerol are virtually ubiquitous in mycoplasmas (86, 236, 241, 301), as in other procaryotes. Phosphatidic acid and lyso compounds may also be present, either as biosynthetic precursors or as breakdown products (264). Sphingolipids, uncommon in bacteria, were found in *Acholeplasma axanthum,* which synthesizes a hydroxyceramide phosphoryl glycerol containing an *O*-acyl group, predominantly 3-hydroxyhexadecanote (197). The phospholipids of the anaerobic *Anaeroplasma* species contain plasmalogens, as in other obligate anaerobic bacteria (160).

Glycolipids. Glycolipids constitute a significant portion of membrane lipids in many, though by no means all, mycoplasmas; *M. hominis* (269) and *S. citri* (86) were reported to lack glycolipids. Nevertheless, small amounts of glycolipids were detected in *S. citri* (S. Razin and S. Greenstein, unpublished data), and a periodic acid-Schiff-positive band migrating in the front of electrophoretic runs made with *S. citri* membranes may represent glycolipids (358). The typical mycoplasmal glycolipids are glycosyl diglycerides containing one to five sugar residues. In *A. laidlawii* the glycolipids are mostly of the monoglucosyl diglyceride and diglucosyl diglyceride types (301), in *M. pneumoniae* they contain both glucose and galactose (153, 301), and in *Acholeplasma modicum* a pentaglycosyl diglyceride containing galactose, glucose, and mannoheptose has been identified (196). A novel glycolipid in *A. laidlawii,* glucolipid X, with a glucose-fatty acid (FA) molar ratio of approximately 1:4 (350) probably has the following structure (M. E. Tourtellotte, personal communication):



A. laidlawii membranes also contain large quantities of phosphoglucolipids, "hybrids" of diglucosyl diglyceride with glycerophosphate or phosphatidic acid, which were accordingly named glycerophosphoryl diglucosyl diglyceride and phosphatidyl diglucosyl diglyceride (302). A glycerophosphoryl monoglucosyl diglyceride has also been identified recently in *A. laidlawii* (350), and a phosphoglucolipid, possibly glycerophosphoryl diglucosyl diglyceride, was detected in *M. mycoides* subsp. *capri* (281). The lipid composition of the *A. laidlawii* membrane has been established in several laboratories with strikingly close results: 25 to 30% phosphatidylglycerol and diphosphatidylglycerol, about 60% glucolipids and phosphoglucolipids, and less than 10% neutral lipids (221, 276, 301, 350). Hence, most membrane lipids in *A. laidlawii* contain carbohydrate moieties.

The ability of *T. acidophilum* to grow at high temperatures and very low pH is reflected in the unique properties of its membrane lipids. The glycolipids and phospholipids of this organism contain glycerol ether residues rather than fatty acid ester-linked glycerides, apparently the result of adaptation to highly acidic conditions, where ester linkages are unstable. The glycerol ethers contain fully saturated isopranoid branched alkyl chains, primarily $C_{40}H_{82}$ and $C_{40}H_{80}$, instead of fatty acids (161, 198, 273) and appear to form unique, symmetrical diglycerol tetraether molecules (159):



Since the length of this molecule approximates 3.6 nm, about the thickness of a lipid bilayer, it has been suggested that the lipid backbone of the *T. acidophilum* membrane consists of a monolayer of these unique molecules. According to this suggestion, the lipid domain of the thermoplasma membrane is formed by hydrocarbon

chains which are covalently linked across the membrane rather than by intercalation of the hydrocarbon chains from two separate and opposite hydrophobic residues. The diglycerol tetraethers may thus provide the membrane with the required structural stability. This concept is in agreement with electron paramagnetic resonance studies by Smith et al. (299), which indicate the thermoplasma membrane to be the most rigid membrane known, and with unpublished findings by P. Ververgaert (cited in reference 159) that *T. acidophilum* membranes cannot be freeze-fractured along their hydrophobic region.

Lipopolysaccharides. As mentioned above, glycosyl diglycerides containing from one to five sugar residues occur in many mycoplasma species. In some of these mycoplasmas, related compounds with more extended oligosaccharide chains were found. They were named lipopolysaccharides, because the presence of oligosaccharide moieties conferred solubility properties resembling those of the classical lipopolysaccharides of gram-negative bacteria (304). Thus, the mycoplasmal lipopolysaccharides are extractable in hot aqueous phenol and form long, ribbon-like structures in negatively stained preparations (199). Moreover, the mycoplasmal lipopolysaccharides are immunogenic, unlike the glycolipids, which are (at most) haptens (303). It should be stressed, however, that in chemical composition the mycoplasmal lipopolysaccharides are totally unrelated to the lipopolysaccharides of gram-negative bacteria. Thus, the lipopolysaccharide of *T. acidophilum* is composed of 24 mannose residues bonded to glucose which is linked to glycerol tetraether (198). Hence, the molecules can be viewed as analogs of glycosyl diglycerides possessing a long polysaccharide chain. Lipopolysaccharides of this type have been found in *T. acidophilum*, where they account for 3% of the cell dry weight, in four *Acholeplasma* species (about 1% of cell dry weight), in the two *Anaeroplasma* species (about 2% of cell dry weight) and in *M. neurolyticum*. None were detected in *M. capricolum*, *M. gallisepticum*, *M. gallinarum*, *M. arthritidis*, and *S. citri* (304). It would be of interest to relate the ability of a mycoplasma to synthesize glycosyl diglycerides with its ability to synthesize lipopolysaccharides. The available data suggest that such a correlation exists, but definite conclusions cannot be drawn as yet. All the mycoplasmal lipopolysaccharides analyzed so far contain both neutral and *N*-acetylated amino sugars in molar ratios varying from 1:1 to 3:1. The neutral sugars are glucose, galactose, and mannose. The amino sugars include fucosamine, an unidentified deoxyhexosamine, galactosamine, and glucosamine. Fucosamine and glucose are the only sugars common to all the mycoplasma lipopolysaccharides, except for that of *T. acidophilum*, which lacks fucosamine (304).

Fatty acids. The fatty acid residues of membrane phospholipids and glycolipids constitute the major portion of the hydrophobic core of the biomembrane, so the physical properties of this core are largely determined by the composition of these residues (240). One of the greatest advantages in taking mycoplasmas as models for membrane studies stems from the fact that these organisms are partially or totally incapable of synthesizing long-chain fatty acids and depend on the growth medium for their supply. This has been exploited most effectively to facilitate controlled alterations in the fatty acid composition of mycoplasma membranes and to study the effects of these alterations on the biophysical and biochemical properties of the membrane, as will be discussed in detail in following sections. The most widely studied mycoplasma for this purpose is *A. laidlawii*. This organism is dependent on an external supply of unsaturated fatty acids but can readily synthesize saturated fatty acids from acetate. Thus, although exogenous fatty acids are incorporated into membrane lipids, forming as much as 50 to 85% of the fatty acids in the membrane, a biosynthetic background of lauric, myristic, and palmitic acids always exists (123, 240). Cerulenin was found to inhibit both the de novo synthesis of long-chain fatty acids and the elongation of medium-chain fatty acids by *A. laidlawii* (262, 277). This inhibitor can thus be applied to reduce the biosynthetic background and to obtain better control of the fatty acid composition of *A. laidlawii* membrane lipids (262). Recent data throw more light on the fatty acid-synthesizing capacity of mycoplasmas other than *A. laidlawii*. A strain of *U. urealyticum* synthesized both saturated and unsaturated fatty acids from acetate (257). All the sterol-requiring *Mycoplasma* (123, 240) and *Spiroplasma* (86; Razin and Greenstein, unpublished data) species tested so far were totally incapable of fatty acid synthesis. More strains of *U. urealyticum* should be tested before any generalizations are made.

Cholesterol. In addition to long-chain fatty acids, most mycoplasmas require cholesterol for growth. The mycoplasmas are the only procaryotes with this requirement, making them unique models for studying the role of cholesterol in biological membranes (246). The cholesterol content of membranes of the sterol-requiring *Mycoplasma* and *Spiroplasma* species is much higher than that found in membranes of the sterol-nonrequiring *Acholeplasma* species, reaching levels comparable to those found in

plasma membranes of eucaryotes (25 to 30% of total membrane lipids [Table 2]). All of the cholesterol found in *Acholeplasma* membranes and most of that found in *Mycoplasma* (269, 295) and *Spiroplasma* membranes (86, 211, 241) is unesterified, despite the excessive amounts of esterified cholesterol in the serum supplement of the growth medium. None of the mycoplasmas tested so far, including the sterol-nonrequiring species, is capable of cholesterol synthesis (86, 301). Furthermore, the unesterified cholesterol incorporated from the medium is generally not esterified or changed in any way (86, 240). The cholesterol esters detected in mycoplasma membranes apparently originate in the growth medium, as their fatty acid composition resembles that of the esterified cholesterol fraction in the serum lipoprotein supplement (269).

Mycoplasmas provide an extremely simple system for the study of serum lipoproteins as cholesterol donors, because, unlike animal cells, they have no endogenous cholesterol synthesis. They also lack the ability to esterify the cholesterol taken up and apparently are incapable of endocytosis of the lipoprotein particles. Thus, in mycoplasmas the process of cholesterol uptake from serum lipoproteins can be studied as a strict membrane phenomenon (246). When purified human serum lipoproteins were added to the mycoplasma growth medium as the sole cholesterol source, the amounts of cholesterol incorporated into the membranes from the LDL were found to be much higher than those taken up from the HDL (295, 296). This result supports the notion that LDL is a far better cholesterol donor than HDL. The much higher molar ratio of unesterified cholesterol to phospholipid in LDL (about 0.8:1) as compared with that in HDL (about 0.15:1) may be responsible, at least in part, for the superior performance of LDL as a cholesterol donor. Cooper et al. (55) suggested that the higher the unesterified cholesterol-to-phospholipid ratio of the cholesterol donor is, relative to that of the membrane, the more effective it is in donating cholesterol. This suggestion is supported by our observation (150) that cholesterol-phosphatidylcholine liposomes with a molar ratio of 0.3:1 served as inefficient cholesterol donors and only permitted poor growth of *M. hominis*, whereas cholesterol-phosphatidylcholine liposomes at a molar ratio of 1:1 and higher acted as very effective cholesterol donors and promoted excellent growth of the organisms. Is cholesterol transferred as a result of a transient contact of the lipoprotein particle with the membrane, or is it the result of a more intimate contact in which the lipoprotein particle fuses and becomes part of the membrane? Experiments with lipoproteins in which the protein moieties were selectively labeled with ^{125}I favor the first alternative by showing that up to 45% of the lipoprotein unesterified cholesterol was taken up by the membranes with little or no concomitant uptake of protein or phospholipid. Moreover, the lipoproteins exposed to the mycoplasmas did not undergo any noticeable degradation apart from their significant depletion of unesterified cholesterol (295, 296).

One problem of great biological significance concerns the mechanism by which cells control the amount of exogenous cholesterol incorporated into their plasma membrane. Studies with mycoplasma cells and membranes have indicated that cholesterol uptake does not depend on metabolic energy and is better characterized as a physical adsorption process (102, 301). Different mycoplasmas vary widely in their cholesterol content when grown with the same supply of exogenous cholesterol (Table 2). Are the differences in cholesterol uptake capacity a result of differences in membrane polar lipid or protein composition, or are they a result of dissimilari-

TABLE 2. *Cholesterol content of representative Acholeplasma, Mycoplasma, and Spiroplasma species*[a]

Organism	Cholesterol (μg/mg of cell protein)			Unesterified cholesterol/membrane phospholipid (μmol/μmol lipid inorganic orthophosphate)
	Total	Unesterified	Esterified	
A. laidlawii	9.1	9.1	0	0.31
M. gallisepticum	45.2	44.0	1.2	1.06
M. mycoides subsp. *capri*	49.1	37.0	12.1	0.83
M. capricolum	53.2	41.5	11.7	0.93
M. hominis	62.0	43.5	18.5	1.15
S. citri	56.3	45.6	10.7	0.92

[a] All the organisms, except for *S. citri*, were grown to an optical density of about 0.3 at 640 nm in Edward medium containing 5% (vol/vol) horse serum. The cells were harvested and washed, and their lipid was extracted and analyzed according to the methods described in references 269 and 296 (unpublished data of S. Rottem). The values for *S. citri*, grown in a somewhat different medium containing 10% (vol/vol) horse serum, were calculated from the data presented in reference 86.

ties in their organization in the membrane? The findings that the ratio of membrane lipid to protein decreases markedly upon aging of mycoplasma cultures (4, 237, 264) and increases with the addition of chloramphenicol (237) were used to show that the amount of cholesterol incorporated into the cell membrane of any specific mycoplasma depends on the phospholipid or polar lipid content of the membrane and is not influenced by variations in membrane protein content (237). That being the case, membranes with a higher specific polar lipid content should be able to incorporate larger quantities of exogenous cholesterol. However, the great differences in cholesterol content of membranes from various mycoplasmas cannot be explained on this basis, since the ratio of polar lipid (phospholipids and glycolipids) to membrane protein is not significantly higher in the cholesterol-rich *Mycoplasma* species than in *Acholeplasma* species (247).

The organization and physical state of the polar lipids in the membrane may influence cholesterol uptake, as has been pointed out by recent studies on the cholesterol-binding capacity of the outer and cytoplasmic membranes of the gram-negative bacterium *Proteus mirabilis* (239). When the bacteria were grown with serum, the cytoplasmic membrane, though located inside the outer membrane, incorporated over four times as much cholesterol per milligram of phospholipid as did the outer membrane. In this case, the two membrane types are known to contain the same phospholipid species, but their fluidity and molecular organization differ markedly (267). Changes in membrane fluidity may affect cholesterol uptake, as suggested by experiments with *A. laidlawii* membranes enriched with either palmitate or oleate (247). The rate constant of cholesterol uptake by the oleate-enriched membrane was significantly higher than that by the palmitate-enriched membrane, but the activation energy of uptake and the amounts of cholesterol bound under equilibrium conditions were about the same. The available data are sufficient to indicate that the content and possibly the molecular organization and physical state of membrane polar lipids can influence cholesterol uptake. The possibility that membrane proteins, lipopolysaccharides, and slime layers may also influence cholesterol uptake by shielding the membrane lipid bilayer from contact with exogenous cholesterol donors warrants further investigation.

Disposition of Lipids in the Membrane

An unequal distribution of the different membrane lipids in the outer and inner halves of the lipid bilayer, known as transbilayer lipid asymmetry, is probably a general property of biomembranes, though experimental data are available only for a few types of membranes (260). Recent evidence supporting this principle in mycoplasma membranes is therefore of great importance. The first indication for exposure of mycoplasma glycolipids on the cell surface came from studies with *M. pneumoniae* where an antiserum to the glycolipids was found to agglutinate the cells (244). More recently, antibodies to a phosphoglucolipid of *M. mycoides* subsp. *capri* were also shown to react with intact cells. The antibodies could be adsorbed by cells as effectively as by isolated membranes, suggesting that a significant part, if not all, of the phosphoglucolipid molecules are in the outer half of the lipid bilayer (281). Glycolipids of erythrocytes and enveloped viruses are located exclusively in the outer half of the membrane (260). The almost equal binding of labeled lectins to intact cells of a variety of *Mycoplasma* species and to their isolated membranes (151) suggests that in mycoplasma also all the carbohydrate-containing membrane components are exposed on the cell surface. Nevertheless, the finding that glycolipids and phosphoglycolipids constitute more than 60% of the total membrane lipids in *A. laidlawii* (221, 276, 301, 350) appears to oppose the notion that all carbohydrate-containing lipids must be externally located.

The external location of at least some of the mycoplasma membrane phospholipids has been suggested by electron microscopy with ferric oxide hydrosols in propanoic acid (284) or polycationic ferritin (283) that bind to negatively charged groups on the membrane surface. The anionic sites appear to be lipid phosphate groups rather than protein carboxyls, since extraction of the membranes with lipid solvents abolished labeling, whereas Pronase treatment increased labeling (283, 284). Striking membrane asymmetry was detected on labeling of isolated membranes of *M. mycoides* subsp. *capri* with polycationic ferritin. The probe was found to bind to one membrane surface only, presumably the outer one (283). On the other hand, *M. hominis* membranes, even when isolated from cells, failed to react with the iron-containing labels unless membrane protein was first digested by Pronase (283). This is in accordance with previous findings showing that the major phospholipid of the *M. hominis* membrane, phosphatidylglycerol, resists hydrolysis by phospholipase C (266) and fails to interact with its specific antiserum (280) unless the membranes are first treated with Pronase, suggesting that membrane phospholipids in *M. hominis* are masked by proteins on both membrane sides.

Information on the localization of phosphatidylglycerol in *A. laidlawii* membranes was recently reported by Bevers et al. (13). Treatment of intact cells with phospholipase A_2 at 37°C caused the degradation of all the phosphatidylglycerol, which constitutes about 30% of the membrane lipids, without causing cell lysis or K^+ leakage. Although by itself this observation suggests that all phosphatidylglycerol is externally located, other findings tend to discredit this explanation. Thus, when linoleic acid-grown cells were kept at 5°C, a temperature at which membrane lipids are still in the liquid-crystalline state, about 50% of the phosphatidylglycerol was rapidly hydrolyzed. The residual phosphatidylglycerol could only be hydrolyzed at elevated temperatures and at much slower rates. Moreover, when membranes isolated from these cells were treated at 5°C, about 70% of the phosphatidylglycerol was hydrolyzed immediately, whereas hydrolysis of the residual phosphatidylglycerol was again strongly temperature dependent. These results led Bevers et al. (13) to suggest the presence of three different phosphatidylglycerol pools: one, consisting of about 50% of the lipid, exposed on the external membrane surface; the second (about 20%) exposed on the inner membrane surface; and the third (about 30%) located in a region protected from the enzyme at low temperatures, probably by association with membrane protein. The fact that all the phosphatidylglycerol was susceptible to hydrolysis on treatment of intact cells at 37°C would then point to rapid transbilayer translocation ("flip-flop") of the lipid at this temperature, probably enhanced by the rapid depletion of the phosphatidylglycerol in the external half of the lipid bilayer.

There can be little doubt that cholesterol taken up from serum lipoproteins, or from any other exogenous source, is first incorporated into the outer half of the lipid bilayer of the mycoplasma membrane. The question which then arises is whether the cholesterol molecules can flip-flop and move from the outer to the inner half of the lipid bilayer and, if they can, how fast this movement is. Evidence that cholesterol is distributed in both halves of the lipid bilayer of mycoplasma was obtained by measuring changes in the spectrum of the polyene antibiotic filipin occurring on its interaction with membrane cholesterol (15). Rapid kinetic studies of filipin binding to intact mycoplasma cells and isolated membranes showed that the initial rates of filipin association with cholesterol were significantly slower with intact cells. Since the initial velocity of filipin association with vesicle-bound cholesterol depends on the accessibility of cholesterol for interaction at the bilayer surface, the rate ratio of filipin association with cells relative to that with membranes may be used as a measure of cholesterol localization in the mycoplasma membrane. These ratios indicated that in membranes of *M. gallisepticum* cholesterol is distributed symmetrically, whereas in *M. capricolum* about two-thirds of the unesterified cholesterol is localized in the outer half of the lipid bilayer (15). The finding that about twice as much filipin was bound to isolated membranes as to intact cells of *A. laidlawii* (67) suggests about equal distribution of cholesterol in the two halves of the bilayer in this organism, too. The presence of about 50% of the membrane cholesterol in the inner half of the lipid bilayer of *M. gallisepticum* has also been indicated by experiments measuring the exchange of [^{14}C]-cholesterol between washed cells or their isolated membranes and HDL (S. Rottem, G. M. Slutzky, and R. Bittman, personal communication). About 50% of the labeled cholesterol in intact cells was readily exchangeable with the lipoprotein cholesterol, against over 90% of the labeled cholesterol in isolated membranes. It can be assumed that the readily exchangeable cholesterol in cells represents that portion of cholesterol located in the outer half of the lipid bilayer, which is exposed to direct contact with the lipoprotein particles.

Although these data indicate that cholesterol moves from the outer to the inner half of the lipid bilayer in mycoplasma membranes, the rate of this transbilayer movement is unknown. We must assume that in growing cells it takes place within the 18- to 24-h period used for growing the organisms with the exogenous cholesterol source. If so, then the flip-flop rate of cholesterol in growing mycoplasmas is much faster than that recorded in artificial phospholipid bilayers, erythrocytes (229), and the influenza virus membrane (260). On the other hand, the flip-flop rate of cholesterol in resting *M. gallisepticum* cells, as measured by the exchange experiments, appears to be much slower than that in growing cells, resembling that recorded for erythrocytes and the influenza virus in similar exchange experiments. This suggests that the flip-flop rate of cholesterol in membranes of growing cells is much faster than that in membranes of nongrowing cells, an intriguing possibility which should be investigated further. However, a more recent study by Lange et al. (158), using a new technique to specifically determine the cholesterol localized on the cytoplasmic surface of the erythrocyte membrane, indicates that the flip-flop rate of the cholesterol in the erythrocyte membrane has a half-life of less than 50 min, a value much smaller than that estimated by them previously (229). Although the discrepancy be-

tween the results obtained in the different exchange experiments has not yet been clarified, the data obtained with growing mycoplasmas strongly favor the fast transmembrane movement of cholesterol.

The functional significance of lipid asymmetry in biomembranes is still obscure. One suggestion is that polar group asymmetry, in conjunction with variations in the fatty acid constituents among lipid classes, results in different fluidities for the two monolayers comprising the bimolecular leaflet (260). The higher freedom of motion of a spin-labeled fatty acid incorporated into membranes of intact mycoplasma cells, as compared with its motion in isolated membranes, was taken to suggest higher fluidity in the outer half of the lipid bilayer (261). Since the various lipid species of *A. laidlawii* may differ significantly in melting temperature (240; Tourtellotte, personal communication) their asymmetrical transbilayer distribution could account for differences in fluidity of the two membrane halves. However, the inner position of most mycoplasma membrane proteins (4, 5) must also be considered in the interpretation of this phenomenon, as membrane proteins can markedly affect membrane lipid fluidity (270).

Regulation of Membrane Fluidity in *Acholeplasma*

A. laidlawii membranes, selectively enriched with different fatty acids, were the first biomembranes shown to undergo a thermal phase transition, a discovery which profoundly influenced the formulation of the fluid mosaic membrane concept (240). The factors which influence the physical state of the lipid bilayer of mycoplasma membranes and the techniques used to determine membrane fluidity have been extensively reviewed and evaluated (240). It is conceivable that for the biomembrane to function, its lipid bilayer must be at least partly in the liquid-crystalline state (201). According to McElhaney (174), up to about one-half of the membrane lipid in *A. laidlawii* may be transformed to the gel state without apparent effects on cell growth, and the existence of less than one-tenth of the membrane lipid in a fluid state is sufficient to support some cell growth and replication, albeit at greatly reduced rates. Once the lipid bilayer totally crystallizes, cells stop growing and the membrane loses its elasticity, so that the cells lyse rather than swell when placed in hypotonic solutions (336); the permeability of the cells to nonelectrolytes, such as glycerol (175), and the valinomycin-induced leakage of K^+ and Rb^+ (335) are reduced to zero; and the activities of some membrane-associated enzymes drop sharply (69, 133, 263, 271). If these and other harmful effects are to be avoided, a mechanism for regulating membrane fluidity becomes essential. It appears that various mycoplasmas have developed different mechanisms for this purpose. Whereas *A. laidlawii* regulates membrane fluidity by adjusting its fatty acid composition, the sterol-requiring mycoplasmas use cholesterol for this purpose.

A most extensive study by McElhaney and coworkers (276–279, 293) provides most of the experimental evidence for the presence of a mechanism for regulating membrane fluidity in *A. laidlawii*. This mechanism acts at various levels of de novo fatty acid biosynthesis and incorporation and elongation of exogenous fatty acids and their utilization for synthesis of the complex membrane lipids. The de novo fatty acid biosynthesis by *A. laidlawii*, in the absence of exogenous fatty acids, was found to yield about an equimolar mixture of saturated C_{14} and C_{16} fatty acids with acetate as a primer and C_{15} and C_{17} fatty acids with propionate as a primer, a mixture which apparently provides optimal membrane fluidity at 35°C (277) *A. laidlawii* is also capable of elongating exogenous fatty acids. Although the substrate specificity of the chain elongation system is strikingly broad, even acting on cyclopropane fatty acids (221, 279), fatty acids with less than 6 to 9 or more than 15 to 18 carbon atoms cannot be elongated. In this way the exogenous fatty acids are elongated to yield only acids having optimal or nearly optimal chain lengths for complex lipid synthesis, so that the fluidity of membrane lipids is maintained within the required range (279). Moreover, the elongation system is markedly influenced by the presence of a second exogenous fatty acid which cannot serve as a substrate for chain elongation, but can be incorporated directly into membrane lipids. In this case the elongation system acts to buffer the physicochemical effect of exogenous fatty acid incorporation by producing a compensatory shift in the average chain length of the elongation product (293). When exogenous fatty acids are provided, *A. laidlawii* most effectively incorporates those saturated fatty acids (C_{14} through C_{16}) which are most similar to the endogenous fatty acids normally produced (276). The organisms could also incorporate and grow well with certain members of the trans-monounsaturated and branched-chain fatty acid series which can provide a moderate level of membrane lipid fluidity (276). Moreover, exogenous medium- or long-chain fatty acids decreased the de novo synthesis of fatty acids, in a manner directed also to regulate membrane fluidity. Thus, an exogenous unsaturated long-chain fatty acid acted to increase the average chain

length of the biosynthesized saturated fatty acids by decreasing the rate of utilization of the shorter chain biosynthetic products in complex lipid synthesis, whereas an exogenous saturated fatty acid depressed the rate of utilization of the long-chain biosynthetic products (293).

Another important factor in modulation of membrane lipid fluidity in *A. laidlawii* is based on the selective incorporation of fatty acids into the 1- and 2-positions of *sn*-glycerol-3-phosphate during phospholipid synthesis (278). High-melting fatty acids exhibit a high affinity for the 1-position, and low-melting fatty acids are bound nearly exclusively to the 2-position. Hence, the enzymes catalyzing the acylation of the 1- and 2-positions appear to recognize the physicochemical properties of their fatty acid substrates, rather than the chemical properties or particular electronic configuration of the fatty acid hydrocarbon chains. This mechanism serves to distribute the high- and low-melting fatty acids fairly homogeneously among the various polar lipid species and in this way decreases the danger of extensive lateral phase separations of the various membrane lipids on decreasing the growth temperature (278).

When taken as a whole, the studies of McElhaney and co-workers (276–279, 293) indicate that in *A. laidlawii* the enzyme systems responsible for fatty acid and complex lipid biosynthesis function to maintain an optimal degree of membrane fluidity. If so, one would expect this mechanism to operate during marked shifts in the growth temperature—a common situation when regulation of fluidity becomes essential. In fact, Rottem et al. (268) showed that decreasing the growth temperature of *A. laidlawii* to 15°C caused a significant increase in the amount of exogenous oleic acid incorporated into membrane lipids. Membranes of *A. laidlawii* grown in the cold (15 or 28°C) were more fluid than membranes of cells grown at 37°C when compared at the same temperature (133, 137, 200, 268), a finding pointing in the same direction. Nevertheless, a significant shifting down in the growth temperature of *A. laidlawii* brought about only minor alterations in the pattern of fatty acids derived from de novo synthesis or chain elongation (279), resulting in only a slight decrease in the average chain length of the fatty acids of membrane lipids (174). It should be stressed that the animal mycoplasmas, including *A. laidlawii*, are usually not exposed to significant temperature changes in their homoiothermic host, but rather may be subjected to marked variations in the composition of fatty acids supplied by the host. The mechanisms for regulating fatty acid composition elucidated by McElhaney and co-workers (276–279, 293) appear quite adequate for this purpose.

Another intriguing problem concerns how the enzymes involved in membrane lipid synthesis select the appropriate fatty acids to maintain membrane fluidity within the optimal range. The work of McElhaney and co-workers (276–279, 293) indicated that the fatty acids are selected on the basis of their physicochemical properties rather than their chemical properties or electronic configurations. Since the binding of a substrate to an enzyme reflects the molecular structure of the ligand, it is difficult to explain the selectivity by enzyme specificity (202). A clear answer to this problem is still unavailable, but an interesting hypothesis was recently proposed by Melchior and Steim (202), according to which the physical state of the lipid bilayer itself determines the concentrations of the various fatty acids available to the enzymes. They provide data showing that the relative affinity of phospholipid bilayers for palmitate as compared with oleate increases with an increase in temperature above the beginning of the phase transition. The more fluid the bilayer, the greater is the binding of palmitate as compared with oleate. The enzyme protein embedded in the lipid bilayer has little or no innate ability to change its selectivity towards various fatty acids but will accept and use the fatty acids supplied to it by the bilayer. In this sense, lipid bilayers would be capable of controlling their own physical state.

Cholesterol as a Regulator of Membrane Fluidity

The total dependence of mycoplasmas on an external supply of cholesterol may be utilized to introduce controlled alterations in the cholesterol content of the membranes, facilitating the analysis of its effects on membrane properties and on cell growth. The successful adaptation of the sterol-requiring *M. mycoides* subsp. *capri* to grow with very little cholesterol (272) provided a useful model system. Adaptation was accomplished by serial transfers of the mycoplasma in a lipid-depleted growth medium containing decreasing concentrations of cholesterol. The cholesterol content of membranes of the adapted strain amounted to less than 3% of the total membrane lipid, as compared with 22 to 26% in membranes of the native strain. The most remarkable difference between the membranes of the two strains was that in only the cholesterol-poor membranes was it possible to demonstrate a thermal phase transition. Differential-scanning calorimetry revealed an endothermic phase transition centered at about 25°C in membranes of the adapted strain, whereas no transition was

discernible in the cholesterol-rich membranes of the native strain. Other techniques, such as fluorescence polarization and freeze fracturing, further confirmed these findings (263). The experiments carried out with the cholesterol-poor *M. mycoides* subsp. *capri* provided the first clear-cut evidence with membranes of growing cells to support the notion that cholesterol regulates membrane fluidity, maintaining an "intermediate fluid condition" during changes in growth temperature or after alterations in the fatty acid composition of membrane lipids (240). In accordance with this supposition, growth of the adapted cholesterol-poor strain was almost completely arrested at 25°C, the temperature at which most of the membrane lipids crystallized, whereas the native cholesterol-rich strain grew well, though at a much slower rate than at 37°C (272). There can be little doubt that the near arrest of growth of the cholesterol-poor strain at 25°C was associated with the steep decline in the membrane-bound ATPase and transport activities which have been shown to occur at this temperature (263). Additional support for the "fluidizing" role of cholesterol in mycoplasma membranes comes from studies carried out some time ago by Smith (300) and more recently by De Kruyff (68). These studies established that to promote mycoplasma growth, the sterol must possess a planar steroid nucleus, a free hydroxyl group at the 3β-position, and a hydrocarbon side chain—the exact structural features required for a sterol to exert a regulatory effect on the fluidity of both artificial membrane systems and mycoplasma membranes (68, 240).

Why are mycoplasmas the only procaryotes dependent on cholesterol for growth? One would tend to associate this requirement with the lack of a cell wall—undoubtedly the single most important property distinguishing the mycoplasmas from all other procaryotes. It has long been suggested that cholesterol increases the tensile strength of the cell membrane of mycoplasmas, thus facilitating their survival and growth without the protection of a rigid cell wall. Cells of the cholesterol-poor *M. mycoides* subsp. *capri*, were, in fact, found to be quite fragile, frequently undergoing lysis even in the growth medium (272). However, this leaves us with the question of why the *Acholeplasma* species and the wall-less bacterial L-forms do not require cholesterol for growth. An answer may be based on the proven ability of cholesterol to act as a regulator of membrane fluidity. As discussed in the previous section, *A. laidlawii* appears capable of regulating its membrane fluidity by changing the fatty acid composition of its membrane lipids, resembling many eubacteria in this respect. The *Mycoplasma* and *Spiroplasma* species, which are totally incapable of fatty acid synthesis, obviously cannot change their fatty acid composition in this way. As to the ability to selectively incorporate exogenous fatty acids, studies carried out with *M. hominis* (264) and *S. citri* (86) showed that at 37°C these mycoplasmas preferentially incorporated palmitate from a mixture of palmitate and oleate in the growth medium. In this case, the incorporation of large quantities of cholesterol into the membrane may be necessary to prevent the membrane from becoming too viscous, even at 37°C. In conclusion, on the basis of the available data it can be argued that the ability of mycoplasmas to incorporate large quantities of cholesterol into their membranes compensates for their inability to regulate membrane fluidity by preferential fatty acid synthesis or incorporation.

MEMBRANE PROTEINS

Solubilization, Fractionation, and Characterization

Peripheral or extrinsic membrane proteins, releasable in water-soluble form by mild treatment of the membranes, constitute a minor fraction of the total protein in mycoplasma membranes (215, 240). Key enzymes, such as ATPase, as well as major membrane antigens and transport carriers, are integral membrane proteins requiring detergents for their solubilization. Prolonged exposure to detergents is likely to lead to conformational changes and loss of the biological activities of purified membrane proteins (240). Recent advances in the methodology of solubilization and fractionation of mycoplasma membrane proteins made by a group working in Uppsala, Sweden, are, therefore, of great importance. The Uppsala group applied the mild detergents Tween 20 and deoxycholate to solubilize proteins of *A. laidlawii* and *S. citri* membranes and fractionated the solubilized proteins by chromatographic gel sieving, agarose suspension electrophoresis, or preparative polyacrylamide or dextran gel electrophoresis (75, 142, 358) in the presence of low detergent concentrations (75) or in the absence of detergent (142). By these procedures, several *A. laidlawii* membrane proteins, including a flavoprotein, an NADH dehydrogenase (75, 142; K.-E. Johansson, personal communication), and a major membrane protein from *S. citri* named spiralin (358), were isolated and partially characterized. The suggestion that spiralin may be responsible for the helicity of *S. citri* (358) is not supported by the work of Townsend et al. (327), who showed that the protein missing from the nonhelical mutant of *S. citri* has a molecular weight of about 39,000, much higher than the value of

26,000 estimated for spiralin (358). Amino acid analyses of some of the isolated proteins failed to reveal an excess of hydrophobic amino acids (358; Johansson, personal communication), suggesting that the hydrophobicity of the molecules depends on the amino acid sequence in the polypeptide chain, as was found for other integral membrane proteins (260).

Glycoproteins. Carbohydrates are only minor components of mycoplasma membranes found in glycolipids, lipopolysaccharides, polysaccharides, and glycoproteins. There is good evidence for the location of the carbohydrate-containing components on the cell surface, so they may play an important role in the interaction of the parasite with the cell membrane of its host. The glycolipid, lipopolysaccharide, and polysaccharide components of mycoplasma membranes were discussed in previous sections. Glycoproteins, though quite common in plasma membranes of eucaryotes, are rare in procaryotes (240), and the search for glycoproteins in mycoplasma membranes is still in its initial stage. Electrophoresis of *M. pneumoniae* membranes in polyacrylamide gels containing sodium dodecyl sulfate revealed a protein band (molecular weight, about 60,000) which stained red with the periodic acid-Schiff reagent (149). This protein, extracted from the membranes with lithium diiodosalicylate, was found (147) to consist of about 80 to 90% amino acids (with the unusual composition of 50 mol% glycine and 20 mol% histidine) and about 7% carbohydrates (mainly glucose, galactose, and glucosamine). Lactoperoxidase-mediated iodination indicated that it is exposed on the external cell surface (149). The possibility that this glycoprotein may be identical with the binding site involved in *M. pneumoniae* attachment to cell surfaces should be investigated. Lithium diiodosalicylate was also applied to *M. gallisepticum* membranes to extract a fraction consisting of about two-thirds carbohydrate and one-third protein (109). This fraction blocked the hemagglutinating activity of *M. gallisepticum* membranes. However, definite identification of this fraction as a glycoprotein could not be obtained, since electrophoresis of the fraction in polyacrylamide gels failed to yield any band which stained with the conventional protein stains and with the periodic acid-Schiff reagent.

An indirect way to look for glycoproteins in mycoplasma membranes is by testing the binding to cells or to isolated membranes of ^{125}I-labeled lectins or by testing for agglutination of the cells by lectins. Lectins were shown to bind to every mycoplasma tested (148, 151), but they agglutinated only a few (282); binding is obviously more sensitive than agglutination (151, 282). To determine whether the carbohydrate groups responsible for the specific binding of the lectins are part of glycoprotein molecules, proteolytic digestion or lipid extraction was applied to the membranes. With most *Mycoplasma* and *Acholeplasma* species, proteolytic digestion increased lectin binding, whereas lipid extraction abolished it (151, 282), indicating the glycolipid or lipopolysaccharide nature of the lectin-binding sites. On the other hand, proteolytic digestion of *M. hominis* (151) and *S. citri* (148) membranes diminished their lectin-binding capacity and their carbohydrate content, suggesting that in this case the lectin-binding sites are parts of glycoprotein molecules. However, glycoproteins could not be detected in either of these two cases by polyacrylamide gel electrophoresis with the periodic acid-Schiff reagent. The possibility that the carbohydrate moieties in *S. citri* or *M. hominis* membranes are parts of macromolecules other than glycoproteins, loosely attached to membrane proteins and released after the removal of the proteins, cannot be ruled out.

Membrane-Bound Enzymes

Electron transport system. As was discussed in a previous section (Energy-Yielding Mechanisms), only in *Acholeplasma* species has the NADH oxidase activity been localized in the membrane (226), but this electron transport system is also very simple and apparently consists of only two components (140, 141, 162, 226; Johansson, personal communication). One of the two, an NADH dehydrogenase, was recently purified from an ethanol extract of *A. laidlawii* membranes by deoxycholate and gel filtration (141). The purified enzyme (estimated to be over 90% pure) did not depend on lipids for activity, supporting previous findings with native or solubilized *A. laidlawii* membranes (69, 214). As expected, the NADH oxidase activity (215) as well as the purified flavoprotein component of this system (143) were localized on the membrane surface facing the cytoplasm.

Adenosine triphosphatase. ATPase has been found associated with the cell membrane of every mycoplasma examined so far, including *S. citri* (148, 211) and *U. urealyticum* (194). In spite of its ubiquitousness, we know very little about its molecular properties, because, unlike other microbial ATPases, it is an integral membrane protein that cannot be detached from the membrane by mild reagents. Detergents inactivate the enzyme rapidly, apparently because of its dependence on membrane lipids for activity (240). The dependence on lipids was first indicated by breaks in the Arrhenius plots of the ATPase activities of *A. laidlawii* (69, 133) and

M. mycoides subsp. *capri* (263) corresponding to the phase transition temperatures of membrane lipids. Direct proof for the dependence of the *A. laidlawii* ATPase on membrane lipids was recently obtained by Bevers et al. (14), who showed that hydrolysis of over 90% of the membrane phosphatidylglycerol by phospholipase A_2 abolished ATPase activity, whereas it had no effect on the *p*-nitrophenylphosphatase and NADH oxidase activities of the membrane. ATPase activity could be restored by liposomes made of phosphatidylglycerol or other acidic phospholipids. The fatty acid composition of the phosphatidylglycerol determined the activation energy of the enzyme and the temperature at which a break in the Arrhenius plot occurred. It is of interest to note that less than 10% of the membrane phosphatidylglycerol (i.e., less than 3% of the total membrane lipids) is required for ATPase activity, supporting the idea that this lipid forms a "halo" of molecules closely associated with the enzyme protein. These and previous data (240) indicate a close resemblance of the mycoplasma ATPase to the (Na^+-K^+)-ATPase of eucaryotic cells with respect to their being integral membrane proteins depending on acidic phospholipids for activity.

Enzymes of lipid metabolism. Most of the enzymes involved in membrane lipid synthesis in mycoplasmas appear to be membrane bound. The list of enzymes provided by Smith (301) and Razin (236) can now be extended. The acyl-CoA:α-glycerophosphate transacylase synthesizing phosphatidic acid, the precursor of membrane phospholipids, was located in the membrane of both *A. laidlawii* and *M. hominis*. Since whole cells showed no significant activity, it appears that this enzyme is located on the inner surface of the membrane. The activation of fatty acids by an acyl-CoA synthetase was, however, localized in the cytoplasmic fraction of these mycoplasmas (264). Another membrane-bound enzyme, a long-chain fatty acyl-CoA thioesterase, has recently been described in mycoplasmas, its activity being highest in *Acholeplasma* species and very low in most of the *Mycoplasma* species tested (271). The *A. laidlawii* enzyme was shown to be an integral membrane enzyme, apparently located on the inner membrane surface, as treatment of intact cells with protease affected its activity only slightly, whereas treatment of isolated membranes inactivated it. Nevertheless, intact cells exhibited high thioesterase activity, apparently due to rapid penetration of the hydrophobic substrates (e.g., palmitoyl-CoA) through the membrane. Arrhenius plots of the thioesterase activity showed breaks at temperatures corresponding to the membrane lipid phase transition, resembling the ATPase activity. In the case of the thioesterase, however, it is possible that the effect of the lipid phase transition on the enzyme activity is due to changes in the solubility of the hydrophobic substrate in the bulk of membrane lipids, rather than to conformational changes in the enzyme molecule itself (271).

Disposition of Membrane Proteins

Transbilayer distribution of proteins. The asymmetrical transbilayer distribution of membrane proteins is even more pronounced than that of membrane lipids (260). Extensive studies on mycoplasma membranes (4, 5, 149), using the lactoperoxidase-mediated iodination system and proteolytic enzymes, indicate that most of the mycoplasma membrane proteins face the cytoplasm, as is probably true for plasma membranes in general (260). The much higher number of particles, representing proteins, on the convex than on the concave fracture face of freeze-fractured mycoplasma membranes (240) supports the above conclusion. The crossed immunoelectrophoretic technique was also applied to localize membrane proteins in mycoplasmas. Solubilized membranes or purified membrane proteins served as antigen, and an antiserum to membranes adsorbed either by intact cells (to remove antibodies to proteins exposed on the outer membrane surface) or by isolated membranes (to remove antibodies to proteins exposed on both membrane surfaces) served as an antiserum. Only one of four purified membrane proteins from *A. laidlawii* was found exposed on the external cell surface by this technique (143), whereas in *M. arginini* one major antigen was found exposed on the outside and two others were apparently completely immersed in the lipid bilayer since antibodies to them could only be adsorbed by Triton-solubilized membrane material (2). The use of membrane reconstitution to study protein disposition has not been successful with mycoplasmas. Reconstituted membranes, produced from mycoplasma membrane material solubilized by sodium dodecyl sulfate or by bile salts, were found to differ radically from the native membranes in disposition of their protein components, apparently due to the denaturation of the proteins by the detergents (215, 236, 238).

Distribution of proteins within the plane of the membrane. Lateral diffusion of membrane lipids and proteins is usually much faster than the thermodynamically unfavorable transbilayer movement, or flip-flop (260). Hence, a homogeneous distribution of lipids and proteins in the plane of the membrane has to be expected under normal growth conditions. However, many studies show that this is not always the

case, since, due to the presence in the membrane of lipids of different melting temperatures, lateral phase separation may take place even within the temperature range enabling normal growth. The differential crystallization of lipids in the bilayer influences the distribution of the proteins partially or wholly immersed in it. Freeze-fracture studies support this notion by showing aggregation of the intramembranous particles during the progressive crystallization of the lipid bilayer of mycoplasma membranes (240). Since in cholesterol-rich membranes phase transition of lipids is prevented, aggregation of intramembranous particles could be demonstrated only in *A. laidlawii* (240) or in the cholesterol-poor *M. mycoides* subsp. *capri* (272). The distribution of the particles on the fracture faces of cholesterol-rich mycoplasma membranes, such as those of *M. gallisepticum* (182), *M. mycoides* subsp. *capri* and subsp. *mycoides* (272; Z. Ne'eman, unpublished data), *M. meleagridis* (116) and *S. citri* (241), was more or less homogeneous.

Whereas freeze fracturing enables the study of the planar distribution and lateral mobility of the proteins immersed within the lipid bilayer, a new technique has recently been used to study the distribution of proteins partly or entirely exposed on the membrane surface (342). The membranes are treated with a biotin-avidin-ferritin complex which specifically links to free amino groups of the exposed membrane proteins. The ferritin component of the complex enables its visualization in the electron microscope. *A. laidlawii* membranes labeled above or below the lipid phase transition showed the labeled sites to be relatively dispersed, whereas in membranes labeled at the mid-transition, low- and high-density patches of label were found. This patching phenomenon was reversible on changing the temperature. As with freeze fracturing, these results indicate that lateral mobility of membrane proteins is affected by the physical state of membrane lipids. However, in contrast to the results of freeze fracturing, where the intramembranous particles remained aggregated below the phase transition, the new data indicate that lateral mobility of membrane proteins may also take place when all the membrane lipid is in the crystalline state, suggesting that this state is not as solid and resistant to diffusion as is widely supposed. Our finding (247) that exogenous cholesterol is incorporated into *A. laidlawii* membranes kept below the phase transition temperature of membrane lipids is in accord with this concept.

Effects of culture age on membrane protein disposition. The process of aging in mycoplasma cultures is accompanied by a marked decrease in the activity of membrane-associated enzymes and transport systems (4, 236, 240) and quite frequently culminates in lysis of the wall-less organisms. There is growing evidence to indicate that these manifestations of aging are associated with alterations in the composition and physical properties of the cell membrane. Thus, the phospholipid (237, 264) and cholesterol (237) contents of membranes from different mycoplasma species were found to decrease most markedly on aging of cultures, resulting in a significantly higher ratio of protein to lipid, a higher density, and reduced fluidity (Table 3). The steep decline in the rate of phospholipid synthesis in aging *M. hominis* cultures (264) and the consequent decrease in uptake of exogenous cholesterol (237) may explain the increased membrane protein-to-lipid ratio, assuming that membrane protein synthesis declines at a lower rate than that of membrane phospholipids. The finding that membranes from aging mycoplasma

TABLE 3. *Changes in membrane composition and properties in aging M. hominis cultures*

Culture absorbance at 640 nm	Viable organisms (colony-forming units/ml)	Membrane lipid-to-protein ratio[a]		Membrane density[b] (g/cm^3)	Lectin binding[c] (μg of concanavalin A/mg of protein)	ATPase activity[b, d]	Membrane fluidity[e] (hyperfine splitting, in gauss)
		μg of lipid phosphorus/mg of protein	μg of cholesterol/mg of protein				
0.10	7.5×10^8	9.5	226	1.162	2.3	3.07	53.9
0.30	1.2×10^9	7.5	158	1.172	1.3	2.00	55.2
0.40	1.7×10^9	6.3	121	1.183	1.1	1.40	56.0

[a] From Razin (237).
[b] From Amar et al. (4).
[c] Unpublished data of A. Amar.
[d] ATPase activity expressed as micromoles of inorganic phosphate released from ATP per milligram of protein in 30 min.
[e] From Rottem and Greenberg (264).

cultures became richer in protein raises two questions. (i) Does the membrane protein composition remain constant during aging? (ii) Are there any changes in the disposition of the various membrane proteins during aging? Experiments on aging *M. hominis* (4) and *A. laidlawii* cultures (A. Amar, S. Rottem, and S. Razin, unpublished data) showed that the overall profile of the major membrane proteins, detectable by polyacrylamide gel electrophoresis, is little affected by aging. Only one protein, exposed on the outer membrane surface, became more prominent on aging of *M. hominis* cultures (4). However, significant changes in the transbilayer distribution of the proteins were indicated by experiments with the lactoperoxidase-mediated iodination system and proteolytic enzymes. These experiments suggested that aging in *M. hominis* cultures is accompanied by a continuous increase in the packing density of the protein molecules on the inner surface of the cell membrane (4). In *A. laidlawii* cultures the number of protein sites on the cell surface available for iodination and exposed to proteolytic digestion decreased markedly with age (Amar et al., unpublished data). It is still not clear whether the changes in protein disposition occurring on aging are associated with altered fluidity of the lipid bilayer. Valinomycin was recently shown to induce similar but much faster changes in membrane protein disposition in *A. laidlawii* cells, suggesting that variations in membrane potential may also affect membrane protein disposition (Amar et al., unpublished data).

TRANSPORT SYSTEMS

Mycoplasmas are attractive subjects for transport studies because the cells are bounded by a single membrane whose lipid composition can be easily altered in a controlled manner. The pronounced influence of the physical state of membrane lipids on a variety of transport phenomena in mycoplasmas, including diffusion of nonelectrolytes, efflux of sugars, and valinomycin-induced Rb^+ and K^+ transport, were discussed in detail previously (240). We can now add to this list transport of glucose by *A. laidlawii* cells (248) and membrane vesicles (220). Recent research has been directed to characterize the components of some of the sugar transport systems and to define transport energetics.

Phosphoenolpyruvate-Dependent Sugar Phosphotransferase System

Some of the fermentative mycoplasmas accumulate sugars by the complex and highly efficient phosphoenolpyruvate-dependent sugar phosphotransferase transport system (PTS) (43, 333). The purification and characterization of the PTS components from *M capricolum* (138, 139) showed the mycoplasma PTS to resemble that of *E. coli* in consisting of membrane-bound sugar-specific proteins (enzymes II) and two soluble nonspecific proteins: a low-molecular-weight protein (HPr) and a higher-molecular-weight protein (enzyme I). The purified *M. capricolum* HPr resembled that of *E. coli* in molecular weight (about 10,000), thermostability, and chromatographic characteristics but differed in having a single histidine residue per molecule and by not showing any significant immunological cross-reactivity with the *E. coli* HPr. The purified enzyme I from *M. capricolum,* which catalyzes the transfer of the phosphoryl moiety from phosphoenolpyruvate to HPr, differed from that of *E. coli, Salmonella typhimurium,* and *Staphylococcus aureus* in molecular properties (139). The mycoplasma enzyme I has a molecular weight of about 220,000 and appears to be comprised of four subunits (two of 44,500 daltons, one of 62,000 daltons, and one of 64,000 daltons), whereas enzyme I from *E. coli* or *S. typhimurium* has a molecular weight of between 70,000 to 90,000 with an unknown number of subunits, and that from *S. aureus* consists of a single polypeptide chain of about 80,000 daltons. The membrane-bound components (enzymes II) of the *M. capricolum* PTS have not been purified and characterized as yet. The enzymes II specific for glucose, fructose, and mannose are constitutive in *M. mycoides* subsp. *capri* (43), whereas in *M. capricolum* the enzyme II for fructose is inducible (139).

Despite the significant molecular differences between the purified PTS components of *M. capricolum* and those of *E. coli* and the wide phylogenetic gap between these organisms, complementation experiments showed that they can replace each other quite effectively. Thus, the mycoplasmal HPr functioned nearly as well as the *E. coli* HPr with *E. coli* enzyme II (138), and the mycoplasmal enzyme I phosphorylated the *E. coli* HPr and vice versa, though the rate in each case was only about 20% of the rate obtained with the homologous components (139). It is not surprising, therefore, that the PTS components from different *Mycoplasma* and *Spiroplasma* species completely complemented each other (138). The complex PTS system in mycoplasmas may have a more fundamental role than that of merely transporting sugars, as is suggested in a recent study by Mugharbil and Cirillo (212) showing that the intracellular level of cyclic 3′,5′-adenosine monophosphate in *M. capricolum* can be regulated by sugars transportable by the organism's PTS. Thus, in the wild type of *M. capricolum,* both glucose and

fructose reduced the intracellular concentration of cyclic 3',5'-adenosine monophosphate, whereas in a mutant which lost glucose-specific enzyme II, glucose no longer affected the intracellular level of the nucleotide. In wall-covered bacteria possessing the PTS system, transportable sugars inhibit the induction of enzymes required for the utilization of alternate energy sources. Inhibition of induction is by combination of a reduction in the intracellular level of cyclic 3',5'-adenosine monophosphate and inhibition of the inducer uptake. The data of Mugharbil and Cirillo (212) show that PTS substrates could fulfil a similar regulatory role in mycoplasmas too.

Sugar Transport in *Acholeplasma laidlawii*

Although *A. laidlawii* species are fermentative and require a metabolizable sugar for growth, they do not possess the PTS system (43, 317). Earlier studies on sugar transport in *A. laidlawii* were hampered by the impermeability of the cells to non-metabolizable substrates, such as α-methylglucoside and 2-deoxyglucose (318), so that metabolizable sugars, such as glucose, maltose, and fructose, had to be used, making it difficult to uncouple transport from assimilation. In spite of this difficulty, these studies (248, 318) sufficed to indicate that glucose permeation into *A. laidlawii* cells occurs via a carrier-mediated process. Definite evidence that this process falls within the category of active transport rather than facilitated diffusion came only after the non-metabolizable analog, 3-*O*-methyl-D-glucose, was found to be taken up by *A. laidlawii* cells against a concentration gradient (315). The isolation, after mutagenesis with nitrosoguanidine, of a mutant defective in glucose and 3-*O*-methyl-D-glucose transport (316) signifies the first example of the application of the genetic approach to elucidating transport systems in mycoplasmas. Furthermore, the Tarshis group in Moscow, U.S.S.R. (78, 79, 220), succeeded in the preparation of membrane vesicles from *A. laidlawii* capable of active sugar transport without metabolizing it. The vesicles, prepared by gentle osmotic lysis of the cells in dilute buffer followed by freezing and thawing, were shown to be sealed and right side out (78). They retained over 60% of the sugar transport capacity of intact cells (220). A somewhat unexpected finding was that exogenous electron donors, such as lactate and pyruvate, did not stimulate glucose transport by the vesicles, apparently because the enzymes responsible for their oxidation are cytoplasmic and are lost during vesicle preparation. It has been suggested, therefore (220), that the membrane vesicles contain enough endogenous electron donors to energize transport. The steep decline in transport activity after several washings of the vesicles supports this idea, though the identity of the endogenous substrates remains unknown.

Tarshis and co-workers (79, 314, 315) put forward the hypothesis that active sugar transport in *A. laidlawii* cells and membrane vesicles is driven by an energized membrane state generated by an electrochemical membrane potential. Inhibition of sugar transport by uncouplers, such as carbonylcyanide *m*-chlorophenylhydrazone (which short-circuits the membrane potential), supports this suggestion (79, 315). The finding that transport of 3-*O*-methyl-D-glucose by *A. laidlawii* cells was also inhibited by *N,N'*-dicyclohexylcarbodiimide, an inhibitor of the membrane-bound ATPase, and by arsenate, an inhibitor of glycolytic phosphorylation, suggested that the energized membrane state is produced by hydrolysis of ATP generated by glycolysis (314). However, *N,N'*-dicyclohexylcarbodiimide and arsenate failed to block glucose transport in *A. laidlawii* membrane vesicles (79). There is no clear reason for the discrepancy between the results obtained with cells and those obtained with membrane vesicles.

FACTORS INVOLVED IN PATHOGENICITY

All members of the class *Mollicutes*, apart from the thermoplasmas, are parasites, and many are pathogens in animals, insects, and plants. Recent literature on mycoplasma-host interrelationships is so voluminous that no effort will be made to cover it completely. This review will focus on pathogenicity factors associated with the mycoplasmas themselves and will not discuss host reactions known to play an important role in the pathogenesis of mycoplasma infections. The reader is referred to several recent reviews and symposia on pathogenicity of mycoplasmas in humans and animals (25, 76, 89, 186, 312, 349) and in plants (21).

Toxic Cell Products

End products of cell metabolism. Hydrogen peroxide, the end product of respiration in mycoplasmas, has been incriminated as a major pathogenic factor produced by mycoplasmas ever since it was shown to be responsible for lysis of erythrocytes by mycoplasmas in vitro (235). It became clear, however, that the production of H_2O_2 does not by itself determine pathogenicity, as the nonpathogenic *A. laidlawii* produces it. Moreover, the swine pathogens *M. hyopneumoniae* and *M. hyosynoviae* produced no more H_2O_2 than did the less path-

ogenic *M. hyorhinis* and *A. granularum* (224), and loss of virulence in *M. pneumoniae* was not accompanied by a decrease in H_2O_2 production (165). As will be discussed at length later, the bulk of the H_2O_2 produced by mycoplasmas in an animal is quickly destroyed by the host catalase and peroxidase activities unless the host is genetically devoid of these enzymes, such as acatalatic mice (29). For the H_2O_2 to exert its toxic effect, the mycoplasmas must adhere close enough to the host cell surface to maintain a toxic, steady-state concentration of H_2O_2 sufficient to cause direct damage to the cell membrane, such as by lipid peroxidation (46). Ammonia is another end product of mycoplasma metabolism which may play a role in mycoplasma pathogenicity when produced in large quantities, as during urea hydrolysis by ureaplasmas (see Organ Cultures as Experimental Systems). Hydrolysis of arginine by mycoplasmas possessing the arginine dihydrolase pathway also yields ammonia as an end product. In this case, however, it is the depletion of the essential amino acid rather than the toxicity of the end product which is to blame for many of the symptoms in cell cultures contaminated by arginine-splitting mycoplasmas (11, 286).

Toxins. So far, a true exotoxin with neurotoxic properties has been found only in cultures of *M. neurolyticum,* the agent of "rolling disease" in mice. The toxin is a thermolabile protein separable from the cells by filtration through membrane filters of 100-nm pore diameter (328). Though some preliminary steps towards its purification were made (323, 328), the toxin has not yet been purified, so that its molecular properties are largely unknown. On filtration through Sephadex G-200 columns it elutes with the void volume (323, 328), suggesting that either it has a molecular weight higher than 200,000 or it is an aggregated form of a smaller protein. When injected intravenously into mice or rats, the toxin rapidly binds to receptors in the brain, probably gangliosides, causing swelling of the capillary endothelium and partial or total occlusion of the capillary lumen (185, 323). Neurotoxic symptoms, not unlike those caused by the *M. neurolyticum* exotoxin, were reported in turkeys inoculated intravenously with *M. gallisepticum.* However, the neurotoxic effects were exclusively associated with viable *M. gallisepticum* cells and not with an exotoxin (323). The mycoplasmas become localized within the arteries of the brain, causing capillary endothelial swelling apparently by gross disturbance of cell permeability (45, 185). In this case, the tropism of *M. gallisepticum* for the arterial walls in the brain and the ability of the microorganisms to reproduce at the arterial site are important factors in pathogenesis. The nature of the toxic factors that damage the capillary endothelium is still a matter of conjecture.

Killed mycoplasma cells or fractions thereof can also be toxic to animals (94). Injection of large quantities of killed *M. fermentans* cells or membranes killed mice with symptoms resembling those of gram-negative endotoxemia, including thymic involution and an increase in the level of the lysosomal β-glucuronidase in the serum (94, 97, 99, 100). These data could suggest that mycoplasmas possess an endotoxin resembling that of the gram-negative bacteria, as was proposed earlier by Villemot et al. (337). Our present knowledge of the mycoplasma membrane composition, however, rules out the presence of any molecules resembling the classical bacterial lipopolysaccharides (see Membrane Lipids). Hence, it is not surprising that the toxemia due to *M. fermentans* cells or membranes differed in some important details from that caused by lipopolysaccharides (99, 100), and the *Limulus* amoebocyte lysate coagulation test, capable of detecting extremely low doses of lipopolysaccharide, remained negative in mice injected with *M. fermentans* (92). Thus, the nature of the toxic factors in *M. fermentans* cells and membranes is still unclear (see also Organ Cultures as Experimental Systems).

The capsular galactan produced by *M. mycoides* subsp. *mycoides* has long been known to prolong mycoplasmaemia and to promote lesions in cattle infected with this organism (170). More recently, injection of calves with the galactan was shown to act similarly to 5-hydroxytryptamine in causing hemorrhages and changes in blood pressure (34), leading to the suggestion that the galactan binds to receptor sites on either blood elements or blood vessel components, causing the release of biogenic amines in the lung.

A step that may bring us closer to the understanding of *S. citri* pathogenicity in plants has recently been made by Daniels et al. (57; M. J. Daniels and C. Barnes, Proc. Soc. Gen. Microbiol. **3**:157–158, 1976). This spiroplasma produces a toxin in vitro which inhibits seed germination, as well as seedling and algal growth, and causes cell injury in leaf tissue. The toxin is a low-molecular-weight (about 200), uncharged compound with vicinal hydroxyl groups and at least one nitrogen atom, implying that it may be an amino sugar. Hopefully, the chemical identification of the toxin will help to elucidate its mode of action.

Adherence to Cell Surfaces

Mycoplasmas rarely invade the bloodstream and tissues. They adhere to and colonize the

epithelial linings of the respiratory and urogenital tracts of infected animals and can thus be regarded as surface parasites. Attachment of the mycoplasmas is firm enough to prevent their elimination by the action of the ciliated epithelium and by the urine. Moreover, the intimate association of the mycoplasmas with their host cell surface provides a nutritional advantage to the parasites. The attached mycoplasmas not only enjoy a higher concentration of nutrients adsorbed onto their host cell membrane (106), but may also utilize the fatty acids and cholesterol of the host membrane itself. Mycoplasmas have been shown to adhere to erythrocytes (176, 306), HeLa cells (177), fibroblasts in monolayer cultures (31), spermatozoa (320), macrophages (145, 146), tracheal epithelial cells (306), tracheal organ cultures (51, 95), and inert surfaces, such as glass and plastic (110, 307, 321). These findings offer a great variety of experimental systems with which to study mycoplasma adherence. For reasons of simplicity and convenience, the adherence of mycoplasmas to erythrocytes, as manifested by either hemagglutination or hemadsorption, has been studied more extensively than the interaction of mycoplasmas with other cell types, though in recent years the tendency has been to use host cells to which the mycoplasmas attach in vivo such as tracheal epithelial cells. The study of mycoplasma attachment to glass or plastic has its merits as well, since it provides a more defined experimental system (110). However, the receptors and binding sites involved in the attachment of mycoplasmas to inert surfaces may be different from those involved in their attachment to cells. The term "receptor site" has become widely accepted to denote the sites on the host cell surface participating in adherence, whereas the term "binding site" is used to specify the sites on the mycoplasma cell membrane responsible for attachment of the parasite to its host.

Nature of receptors. The chemical nature of the receptors on the eucaryotic cell surface responsible for mycoplasma attachment has been established for only a few of the mycoplasma species showing adherence properties. Since attachment of *M. pneumoniae, M. gallisepticum,* and *M. synoviae* is affected or even abolished by pretreatment of the host cells with neuraminidase (103, 177), it is the accepted view that these mycoplasmas attach to sialic acid moieties on the host cell surface. Supporting this is the recent finding that glycophorin, the erythrocyte membrane protein carrying almost all the sialic acid moieties of the erythrocyte, inhibited *M. gallisepticum* attachment to erythrocytes (M. Banai, I. Kahane, S. Razin, and W. Bredt, unpublished data). Nevertheless, it is not clear whether the sialic acid moieties are the only receptors for these mycoplasma species. Recent studies by Powell et al. (228) and Engelhardt and Gabridge (77), using labeled *M. pneumoniae* cells to quantify adherence, showed that treating the tracheal epithelial cells with neuraminidase decreased attachment by about 50 to 65%, but did not abolish it. This residual "background attachment" (77) may involve receptors other than sialic acid. The finding that the poor attachment of the avirulent *M. pneumoniae* strain to hamster epithelium was not affected by neuraminidase treatment (52) points in this direction as well. Gabridge et al. (95) also found that the attachment of isolated *M. pneumoniae* membranes was not readily affected by neuraminidase treatment of the tracheal explants. It can be argued, however, that by isolating the cell membranes, new binding sites were exposed which could attach to receptors other than sialic acid. All the above studies suffer from the same deficiency: lack of data on the number of sialic acid residues left on the host cell membrane after neuraminidase treatment. Recent studies in our laboratory (M. Banai, unpublished data) show that the most extensive treatment of human erythrocytes with neuraminidase failed completely to remove the sialic acid residues, as determined by gas-liquid chromatography. The treated erythrocytes still possessed about 5% of their original sialic acid content and exhibited about 20% of their original capacity to bind *M. gallisepticum.* Hence, the residual capacity of the treated eythrocytes to attach to *M. gallisepticum* could be attributed to residual sialic acid moieties. Another interesting question is whether the predilection of mycoplasmas for certain mucosal surfaces is dictated solely by the availability of the specific receptor sites. In the case of the mycoplasmas attaching to sialic acid receptors, the answer is clearly negative, as sialic acid residues are found essentially in all mucosal surfaces in the body.

No information is available on the chemical nature of the receptors for mycoplasmas other than *M. pneumoniae, M. gallisepticum,* and *M. synoviae.* The erythrocyte receptor sites for *M. dispar* were insensitive to neuraminidase, proteolytic enzymes, and periodate (128). Similarly, the attachment of *M. pulmonis* to mouse macrophages was not affected by pretreatment of the macrophages with neuraminidase, trypsin, chymotrypsin, or glutaraldehyde (146), suggesting that the receptor sites are neither sialic acid residues nor membrane proteins. On the other hand, the receptors on the HeLa cells responsible for attachment of *M. hominis* and *M. salivarium,* though resistant to neuraminidase, were inactivated by proteolytic enzymes and For-

malin (177), suggesting that they are proteins. It thus appears that different cells may have different receptors specific for various mycoplasmas.

Nature of binding sites. Available data do not allow us to ascertain the chemical nature of any of the binding sites on the cell membrane of adhering mycoplasmas. Pretreatment of mycoplasmas with neuraminidase does not affect their ability to adhere, so sialic acid can be ruled out as a binding site (177). In fact, sialic acid is rarely found in procaryotes (154) and has not been detected in mycoplasmas. Detection of neuraminidase activity in *M. gallisepticum* (290) raises the possibility that in this mycoplasma the neuraminidase acts as a highly specific binding site for the sialic acid receptors on the host cell surface. If true, this would require the enzyme to be located on the mycoplasma cell surface and the detachment of the adhering mycoplasmas after hydrolysis of the sialic acid residues, but no evidence regarding this is available.

Mostly indirect but satisfactory evidence is available for the protein nature of the binding sites in *M. pneumoniae* and *M. gallisepticum*. Heat or Merthiolate abolished the ability of *M. gallisepticum* to agglutinate erythrocytes (103). Trypsin or heat inactivated the binding sites of *M. pneumoniae* responsible for adherence to tracheal epithelial cells (306), erythrocytes, or polystyrene beads (110). Similarly, trypsin detached the sheets of *M. pneumoniae* and *M. gallisepticum* growth on plastic or glass (177, 307). The ability of *M. pneumoniae* to adhere, lost by trypsin treatment, could be restored by incubating the treated cells in a growth medium for 4 h at 37°C, but not at 4°C. Chloramphenicol, mitomycin C, or ultraviolet irradiation inhibited restoration (110). Hu et al. (136) progressed further by identifying a major membrane protein exposed on the surface of *M. pneumoniae* cells that is required for the attachment of the mycoplasmas to tracheal cells. Similarly, the binding site of *M. dispar* to sheep and bovine erythrocytes appears to be either a protein or associated with a protein moiety (128). Nevertheless, *M. hominis* and *M. salivarium* sheets adhering to plastic could not be detached by trypsin (177), nor did trypsin treatment of *M. pulmonis* affect its attachment to mouse macrophages (146). Hence, as with the receptor sites, generalizations cannot be made as to the chemical nature of the binding sites.

The polarity exhibited by *M. pneumoniae, M. gallisepticum,* and *M. pulmonis* cells adhering to inert surfaces (23, 24) or to cells (51, 53, 361) through their specialized tip structures or blebs, would suggest a high concentration of binding sites on the surface of these structures. Evidence for this suggestion must await the isolation and chemical characterization of these structures. The finding that isolated *M. pneumoniae* membranes attach poorly to tracheal ring cells, as compared with metabolically active cells, was taken by Hu et al. (135) to indicate that cell motility and proper orientation of the terminal structure fulfil an important role in mycoplasma attachment. Nevertheless, scanning electron microscopy has recently shown that individual *M. pneumoniae* filaments attach to glass (213) or to erythrocytes (H. Brunner, personal communication) throughout their entire length, suggesting that binding sites are also available on membrane regions other than those of the tip structure. The perpendicular orientation of *M. pneumoniae* attaching to ciliary epithelium may be explained by the close packing of the cilia, which would hardly permit parallel alignment of the mycoplasmas (18).

Are all the mycoplasmas within a population able to attach with the same avidity to the host cell? Our recent results (Banai et al., unpublished data) give a negative answer to this question. The *M. gallisepticum* cell population remaining in the supernatant fluid after exposure to erythrocytes showed a much poorer capacity to attach to erythrocytes during a second attachment test, indicating an unequal distribution of binding sites among cells within the same population.

Factors affecting adherence and nature of bonds. The nature of bonds participating in mycoplasma adherence appear similar in the various experimental systems. The adherence of *M. pneumoniae* to tracheal epithelial cells (228) or that of *M. gallisepticum* to erythrocytes (Banai, unpublished data) followed first-order kinetics. Engelhardt and Gabridge (77) demonstrated that at any given time less than 10% of the viable *M. pneumoniae* organisms attach onto the tracheal ring explants, suggesting the reversibility of the adherence process. As would be expected from a reaction depending on the frequency of impact of particulate matter, adherence of mycoplasmas to cells is usually temperature dependent (110, 176). Wall-covered bacteria adhere to eucaryotic cells mostly by London-van der Waals forces (106). However, to activate these attractive forces, the repulsive electrostatic charges on the bacterial and host cell surfaces must first be overcome. It has been suggested that pili facilitate attachment of gonococci to epithelial cells by overcoming these repulsive electrostatic charges (120). The cell surface of mycoplasmas carries a net negative charge (265, 283) and has no pili, so it is not clear how the adhering mycoplasmas counteract the electrostatic barrier. The available data in-

dicate that at least some of the bonds attaching mycoplasmas to the host cell surface are neither electrostatic bonds nor ion-bridging bonds, as changing the ionic strength, increasing the pH of the medium from 6 to 8, and chelating of divalent cations showed little effect on attachment of *M. pneumoniae* (110, 307) and *M. gallisepticum* (Banai et al., unpublished data) to erythrocytes, glass, or plastic surfaces. Obviously, these data do not rule out the participation of ionic bonds in mycoplasma adherence, but they indicate that these cannot be the sole bonds involved.

Importance of adherence to pathogenicity. The intimate association between adhering mycoplasmas and their host cells provides a situation in which local concentrations of toxic metabolites can build up and cause cell damage. Thus, the H_2O_2 excreted by the mycoplasmas attached to the host cell surface may attack the cell membrane without being rapidly destroyed by catalase or peroxidase present in the extracellular body fluids. Hydrolytic enzymes produced by the mycoplasmas may damage the host cell membrane as well. The neuraminidase of *M. gallisepticum* may attack the sialoglycoproteins on the host cell surface, modifying the cell charge and consequently affecting contact inhibition and cell adhesiveness (290). The protease and phospholipase activities associated with mycoplasma membranes (240) may affect the protein and lipid components of the host cell membrane. The high rate of exchange of [^{3}H]-oleic acid between labeled lipids of *M. pneumoniae* and tracheal epithelial cell membranes (228) may be indicative of phospholipase action. It can also be suggested that adhering mycoplasmas may take up cholesterol from the host cell membrane and in this way deplete it of an essential component.

The hypothesis that adherence leads to fusion of the cell membrane of the parasite with that of its host has been recently promoted by Gabridge et al. (95). If fusion does occur, then a wide variety of potentially cytotoxic proteins (enzymes) and lipids can be introduced directly into the host cells without being contained in the typical, protective vesicle associated with normal phagocytosis and particle uptake. The new "patch" on the epithelial cell membrane may provide a relatively weak area which could leak ions or essential metabolites (95). Although the fusion hypothesis is attractive, the only evidence available to support it is based on electron microscopy suggesting the fusion of *M. gallisepticum* with erythrocytes (6). Even in this case, only a small proportion of the mycoplasmas fused, and the factors influencing this process were not defined. Essentially all the other micrographs published so far of mycoplasmas adhering to eucaryotic cells show a distance of about 10 nm separating the mycoplasma membrane from that of the host (e.g., 18, 128, 145, 355), a finding which does not support fusion.

A specific case in which adherence may play an important role in pathogenicity is that of mycoplasma adherence to spermatozoa. The possibility that genital mycoplasma adhering to spermatozoa affect their fertility was first raised by Taylor-Robinson and Manchee (320) and later promoted by Gnarpe and Freiberg (107) and Fowlkes et al. (83), who demonstrated, by scanning electron microscopy, mycoplasmas adhering to human spermatozoa of low fertility. Although there is still no direct evidence for mycoplasma causing low fertility in human spermatozoa, the preincubation of mouse spermatozoa with *M. pulmonis* reduced both fertilization and subsequent embryonic development of mouse eggs *in vitro* (85). Spermatozoa may also act as vehicles transporting adhering pathogenic mycoplasmas deep into the female genital tract during fertilization (O. H. Stalheim and J. E. Gallagher, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, G9, p. 132).

To conclude, the ability of mycoplasmas to adhere to their host cells assists them in the disease process, and the identification and isolation of the mycoplasma surface components responsible for adherence may be used in the future to develop highly specific vaccines. Nevertheless, it should be stressed that the ability of mycoplasmas to attach to cells in vitro does not necessarily indicate pathogenicity in vivo and vice versa. Nonpathogenic mycoplasmas, such as *M. salivarium,* were shown to adhere to cell surfaces in vitro, whereas some pathogenic mycoplasmas, such as the two *M. mycoides* subspecies, failed to adhere to erythrocytes in vitro (176).

Organ Cultures as Experimental Systems

Tracheal organ cultures were introduced as experimental model systems for studying pathogenicity of respiratory mycoplasmas in 1969 independently by Butler (32) and by Collier et al. (54). Later, the oviduct was added to study pathogenicity of genital mycoplasmas (188, 309, 311, 319). Organ cultures present several advantages over intact animals in pathogenicity studies. Most important is the ability to continually observe the effects of the parasite on its natural "target cell" under controlled conditions. Ciliary activity, which is hormone independent, continues for several days or even weeks, permitting continuous monitoring of injury and viability of the epithelial cells. The organ culture facilitates studying toxic factors produced by the parasite

and metabolic disturbances caused by them (134). The pathogenicity of mixed mycoplasma and virus infections and their control by chemotherapy (32, 249) can also be conveniently studied in organ cultures. The use of organ cultures most probably eliminates hormonal effects and immunological responses, as well as age, weight, and sex differences, factors which may affect the response of the intact animal to the parasite. Though this simplifies the system, it is also its most serious deficiency, since it can be argued that these factors, not functioning in organ cultures, may profoundly influence the response of the target cells to the parasite in the intact animal (51, 52).

Localization of mycoplasmas in organ cultures. That mycoplasmas can grow in tracheal and oviduct organ cultures has been demonstrated by many authors (32, 33, 41, 54, 188, 311, 319), though a period of adaptation to the explant conditions is sometimes necessary before satisfactory growth will occur (33). The organ culture usually supports better growth than the conditioned medium from which the organ explants have been removed, probably because of the higher concentration of nutrients adsorbed onto the organ cell surfaces. Electron microscopy showed that elongated *M. pneumoniae* cells adhered to the host cell membrane through their terminal structure. Although the mycoplasmas sometimes occupied intercellular spaces, they were never seen inside the epithelial cells (51). The importance of adherence to mycoplasma pathogenicity (see Adherence to Cell Surfaces) has been supported by organ culture studies. The avirulent *M. pneumoniae* strain, defective in its adherence properties, failed to induce damage in tracheal explants (51, 134). Likewise, the cytopathic effect of *M. dispar* on bovine tracheal explants was expressed only when the mycoplasmas attached to the epithelial cell surface (324). *M. gallisepticum,* shown to adhere firmly and colonize the ciliated cell surfaces of chicken tracheal explants, exerted a much earlier and more pronounced cytopathic effect than *M. gallinarum,* which failed to develop an intimate association with the cells but grew as well as *M. gallisepticum* in the tracheal explants (1).

Manifestations of tissue damage. The dulling of ciliary activity followed by complete ciliostasis is the most pronounced manifestation of injury to the tracheal or oviduct explants. Ciliostasis has therefore been extensively used to monitor tissue damage (32, 41, 51). Sections of tracheal explants infected by *M. pneumoniae* and examined by light and electron microscopy exhibited cytoplasmic eosinophilia, vacuolization, and nuclear swelling followed by protrusion of some epithelial cells into the lumen. With the progress of infection, the cilia were distorted and lost, followed by the desquamation of the superficial epithelial cells (51). Similar symptoms were observed on infection of bovine oviduct cultures with ureaplasmas (311). Scanning electron microscopy has produced dramatic electron micrographs of damaged ciliary epithelium in infected organ cultures by permitting visualization of large surface areas at high resolution (311, 357). The histological manifestations of tissue damage reflect pronounced alterations in the metabolism of the infected organ cultures. Thus, the oxygen consumption levels (93), tetrazolium reduction rates (98), and ATP content (98a) of tracheal explants infected with *M. pneumoniae* decreased markedly, facilitating the quantification of tissue damage. On the molecular level, infection of hamster tracheal explants with *M. pneumoniae* caused a marked decrease in RNA and protein synthesis (134), but the turnover rates of these macromolecules resembled those of uninfected controls, suggesting that the reduction in the rates of RNA and protein synthesis did not result from increased activity of hydrolytic enzymes (135).

Nature of toxic factors. The nature of the factors causing the damage to organ explants infected by mycoplasmas is still unclear. Only in the case of the bovine oviduct infected by ureaplasmas has the toxic factor been identified with some degree of confidence. Stalheim and Gallagher (310) could duplicate the pathological effects of ureaplasma infection by adding ammonium sulfate or urease to uninfected organ cultures, indicating that the ammonia produced by the ureaplasmas from urea in the culture medium is the toxic factor. This conclusion is in accord with previous findings by the same authors (311) that washed or disrupted ureaplasma cells may still cause ciliostasis, whereas heating of the ureaplasmas to 56°C for 30 min diminished, and boiling abolished, their cytopathic activity.

As to hydrogen peroxide, only in the case of *M. mycoides* subsp. *capri,* which produces high quantities of it, could the damage to the organ culture be delayed by the addition of catalase (41). Catalase did not offer any protection to organ cultures infected by *M. gallisepticum* (41), *M. dispar* (129), and *M. pneumoniae* (134). In all cases, including that of *M. mycoides* subsp. *capri,* a toxic factor could not be demonstrated in the spent organ culture medium from which the mycoplasmas had been removed by centrifugation or filtration (324). Hence, if peroxide is indeed a toxic factor, as is probably the case in *M. mycoides* subsp. *capri,* it can exert its effect only during the close association of the myco-

plasmas with the host cell membrane.

Since the search for toxic soluble by-products failed in almost all cases, attention has been directed to the possibility that the mycoplasma cell components themselves are toxic to organ cultures, an idea promoted mostly by Gabridge et al. (96). Isolated *M. pneumoniae* membranes, but not the cell cytoplasm, induced the characteristic pathological manifestations, including ciliostasis, in hamster tracheal explants. Membrane toxicity was attributed to its lipid component, ruling out the possibility that membrane-associated enzymes are responsible for tissue damage. S. E. Singer and M. G. Gabridge (personal communication) propose that cytotoxicity may result from the incorporation of mycoplasmal lipids into the epithelial cell membrane causing perturbation of the lipid bilayer and leakage of ions and metabolites. This idea is, however, strongly challenged by Hu et al. (135) and by Powell et al. (228), who failed to reproduce the results of Gabridge and, therefore, insist that only metabolically active *M. pneumoniae* cells are able to damage the organ culture. Singer and Gabridge (personal communication) try to reconcile these contradictory results by suggesting that the isolated membrane dispersions prepared by Hu et al. (135) differed from theirs and were not applied to the organ cultures in sufficient quantities: large quantities of membranes (at least 50 μg of membrane protein per ml) were needed to exert cytotoxic effects in organ cultures, a finding to be expected if membrane toxicity is indeed based on the lipid component rather than on catalytic activities associated with the membranes. Although it is still too early to decide who is right, both sides agree that viable *M. pneumoniae* cells, by virtue of their marked tendency to attach to epithelial cells, are much more effective in causing tissue damage than are dead cells or isolated membranes.

As for mycoplasmas other than *M. pneumoniae,* membranes of *M. fermentans* toxic to mice had no effect on hamster tracheal organ cultures (96), whereas membranes of the nonpathogenic *A. laidlawii* were cytopathic, with the toxicity attributed to the protein component of the membranes (Singer and Gabridge, personal communication).

Correlation of pathogenicity in vitro with that in vivo. The freedom of organ cultures from host responses raises two major questions: (i) is the host specificity, characteristic of many mycoplasma infections, retained in organ culture systems, and (ii) does the ability of a mycoplasma to induce cytopathic effects in organ cultures correspond with its ability to cause disease in the intact animal? Although cytopathogenicity of *M. gallisepticum* to tracheal explants of chicken, but not of human, origin (33) and the reverse behavior of *M. pneumoniae* (52) suggest the preservation of host specificity in organ cultures, the abilities of *M. pneumoniae* to cause ciliostasis in bovine oviduct explants (309) and of *M. mycoides* subsp. *capri* to damage chicken tracheal explants (41) suggest that host specificity is weakened in organ cultures. As for the second question, the answer is far from clear. On the one hand, Thomas and Howard (324) found the bovine respiratory pathogen *M. dispar* to be also cytopathic to bovine tracheal explants, and Hu et al. (134) showed that the loss of virulence of an *M. pneumoniae* strain in hamsters is accompanied by loss of cytotoxicity to hamster tracheal explants. On the other hand, there are reports showing no correspondence at all. Thus, *M. hyponeumoniae,* the agent of porcine enzootic pneumonia, was not pathogenic to pig tracheal explants, whereas the much less pathogenic *M. hyorhinis* and *A. granularum* exhibited distinct cytopathic effects (224, 225). These results could have been influenced, however, by the much poorer growth of *M. hyopneumoniae* in vitro, as compared with that of the other swine mycoplasmas. Similarly, the extremely poor growth of ureaplasmas in vitro may explain the failure of these organisms to produce cytopathic effects in organ cultures derived from animals susceptible to infection by these mycoplasmas (319, 324). Ureaplasma growth in bovine oviduct explants, in which cytopathic effects were reported, however, was about 100 times greater than that normally recorded in cell-free media (311). The finding (310) that ammonia is responsible for the cytopathic effects of ureaplasmas raises another question: does ammonia play a role in the pathogenicity of ureaplasmas in vivo? In the urinary tract, with high concentrations of urea provided in the urine, this is certainly possible, and, in fact, ammonium-magnesium-phosphate stones have been produced by infecting the rat bladder with ureaplasmas (90). On the other hand, ureaplasmas have also been shown to cause pneumonia and mastitis in animals, damaging organs which contain very little, if any, urea. Stalheim and Gallagher (310) suggest that compounds other than urea may be possible substrates for the ureaplasmal urease, yielding the toxic ammonia. Ammonia can also be produced by degradation of amino acids, such as by the activity of the L-histidine ammonia-lyase detected in ureaplasmas (3).

MYCOPLASMA VIRUSES

The finding by Gourlay (111) that even the smallest procaryotes are infected by viruses and

thus have parasites of their own was quite exciting. Though one might expect that the viruses infecting the wall-less mycoplasmas differ from classical bacteriophages and more closely resemble animal cell viruses, it now appears that mycoplasma viruses resemble bacteriophages in some properties and animal viruses in others. Several reviews on mycoplasma viruses are available (47, 113, 179, 181, 352).

Thus far, viruses have been propagated only in *A. laidlawii*, so most of the available information concerns viruses infecting this mycoplasma (Table 4), designated by Gourlay as MV-L1, MV-L2, and MV-L3 (MV = *Mycoplasmatales* virus, L = *laidlawii*). Morphological evidence for virus-like particles is available for several *Mycoplasma* species (126, 251). The presence of satellite DNA in several *Mycoplasma* species was interpreted as pointing in the same direction, though its identification with plasmids is more likely (see Genome). Plaque formation on a lawn of an *M. pulmonis* strain was reported (115), but failure to propagate these plaques and detect viruses in them indicates that they are not caused by viruses. In spite of the fruitless efforts thus far to isolate viruses from *Mycoplasma* and *Ureaplasma* species, the recent report of plaque formation by the SV-C3 virus of *S. citri* (47) indicates that continued search for appropriate indicator lawns may prove rewarding, as it is difficult to believe that *Mycoplasma* and *Ureaplasma* species are totally free of virus infection. Nevertheless, the much smaller genome sizes of the *Mycoplasma* and *Ureaplasma* species led Gourlay (113) to suggest that viruses in *Mycoplasma* and *Ureaplasma* species, if existing, possess properties dissimilar to the acholeplasma and spiroplasma viruses, and hence new methods of virus isolation may have to be evolved.

Properties of *Acholeplasma laidlawii* Viruses

The three viruses infecting *A. laidlawii* differ considerably in structure, chemical composition, and mode of replication. The total unrelatedness of these viruses is also expressed by the lack of any antigenic relationship, so that an antiserum against one will not inhibit the others and *A. laidlawii* clones made resistant to one virus will still be sensitive to infection by the other two viruses (112, 113). MV-L1, the virus studied in most detail, is a rod-shaped virus consisting of a single-stranded, covalently closed circular DNA molecule covered by a cylinder of protein subunits arranged in helical symmetry. The MV-L1 chromosome resembles that of the single-stranded DNA filamentous and icosahedral phages (71) and is capable of providing information for the synthesis of six to eight proteins. Four proteins were detected by polyacrylamide gel electrophoresis of the purified virus (180). There is still some controversy as to the true morphology of MV-L1. Whereas all workers agree that one end of the rod-shaped particle is rounded, Liss and Maniloff (169) claim that the other end of the virion (the basal end that presumably makes contact with the cell membrane) is relatively flat, giving the rod a bullet shape. On the other hand, Gourlay (113) claims that

TABLE 4. *Some properties of mycoplasma viruses*[a]

Virus	Host[b]	Morphology	Density (g/cm^3)	Nucleic acid
MV-L1 (group 1)	*A. laidlawii*	Rods, 14–16 nm by 70–90 nm	1.37 (in CsCl)	Single-stranded circular DNA, 2×10^6 daltons
MV-L2 (group 2)	*A. laidlawii*	Enveloped spheres, 80 nm average diameter	1.26 (in CsCl)[c] 1.19 (in sucrose)	Double-stranded DNA[d]
MV-L3 (group 3)	*A. laidlawii*	Polyhedron with short tail; 57- by 61-nm head, 9- by 25-nm tail	1.48 (in CsCl)	Double-stranded DNA
SV-C1	*S. citri*, CSO, SRO	Rods, 10–15 by 230–280 nm	Not known	Not known
SV-C2	*S. citri*	Polyhedron with long tail; 52–58-nm head, 7- by 80-nm tail	Not known	Not known
SV-C3	*S. citri*, CSO, SRO, SMCA	Polyhedron with short tail; 37- by 44-nm head, 7- by 16-nm tail	Not known	Not known
spv-1	SRO	Polyhedron with tail resembling SV-C3	1.48 (in CsCl)	DNA

[a] For references, see Maniloff et al. (179) and Cole (47).
[b] CSO, Corn stunt organism; SRO, sex ratio organism; SMCA, suckling mouse cataract agent.
[c] A. L. Watkins (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, G24, p. 134).
[d] J. Maniloff (personal communication).

the true shape of the complete virion resembles that of a bacillus, with both ends rounded. The bullet-shape forms are, according to this view, the result of degradation of one tip of the rod.

MV-L2 is a spherical enveloped virus, the study of which has been limited due to problems in collecting and purifying enough viral material. There are indications, based on electron microscopy, that its DNA is double stranded (179), but its form, size, and base composition are still unknown. Thin sections of the virus show its envelope to have the trilaminar unit membrane shape without surface projections (113). The low density of the virus (Table 4) and its high sensitivity to inactivation by organic solvents, detergents, and heat indicate that its envelope is made of lipids and proteins. However, nothing is known of the lipid composition of the virus and its relationship to membrane lipids of the host. The virus interior appears fibrillar with no evidence for a rigid internal helical or icosahedral capsid structure, suggesting that the core of the virion may simply consist of a folded double-stranded DNA molecule with some packing proteins.

More information is available on MV-L3, the naked tailed polyhedron (Table 4). This virus is composed of about 35% double-stranded DNA, 63% protein (consisting of three major and two minor polypeptides), and 1.8% fucose. It is not clear whether the fucose forms part of a viral glycoprotein or whether it is attached to the viral DNA (101).

Virus Adsorption

The details of the replication cycle of MV-L1, or its variant MVL51, have been unraveled mostly through the work of Maniloff and his group. Very little is known on the replication of the other two *A. laidlawii* viruses. The initial step in viral infection is its adsorption to the host cell surface. This stage in mycoplasma virus replication is of particular interest, since, like animal viruses, mycoplasma viruses bind directly to the host plasma membrane, whereas the classical bacteriophages bind to the bacterial cell wall, flagella, or pili. Unfortunately, information on this subject is very scarce and comes mostly from the work of Fraser and Fleischmann (84) on the adsorption of MV-L1 to *A. laidlawii* cells. The adsorption, which followed pseudo-first-order kinetics, resembled other viral systems, being ionic in character. The pH optimum for adsorption, about 6.0, seems to militate against membrane phospholipids' participating in viral binding and favors the imidazole moiety of histidine (pK of 5.99 at 25°C) taking part in this process. Calculations based on the kinetics of the adsorption process suggested that nearly every collision between cell and virus results in adsorption. The number of effective adsorption sites per cell was calculated to be about 10 (84). This number is three to five times higher than the number of sites on the inner membrane surface to which parental viral DNA molecules can bind and actually participate in DNA replication (59). Though electron microscopy showed numerous MV-L1 particles adsorbed to the external surface of infected *A. laidlawii* cells, forming palisade-like arrays (206), in all likelihood these particles represent viruses extruded from the cells after virus maturation.

Recent work by Putzrath and Maniloff (230) suggests that only about 10% of the potential MV-L2 virus-cell collisions result in virus adsorption, as against 100% found for MV-L1 (84). No further information is available on the attachment of MV-L2 to the host, and essentially nothing is known on the nature of the binding of MV-L3 to its host apart from indications, based on electron microscopy, that the virus attaches with its short tail (113).

Deoxyribonucleic Acid Penetration

After the attachment of the virus to the cell, the viral DNA enters through the cell membrane. The fact that mycoplasmas are wall-less makes it unnecessary for their viruses to develop the elaborate injection mechanism found in many bacteriophages. Yet, the tails of MV-L3 and the spiroplasma virus-like particles SV-C2 and SV-C3 (Table 4) are reminiscent of bacteriophage tails but show no evidence for contractility, a characteristic feature of the classical bacteriophage tail (47). Moreover, MV-L1, MV-L2, and the spiroplasma virus-like particle SV-C1 have no tails at all. How then does the viral DNA enter through the membrane? There is no evidence for engulfment or "phagocytosis" of the virus particles by the mycoplasma cells. In this respect, the mycoplasma viruses seem to differ from animal cell viruses. The finding of hollow rods of MV-L1 led to the suggestion (113) that the viral DNA is released into the cell after breaking of the tip of the virus attached to the cell, but this still does not answer the above question. Treatment of the cells before infection with chloramphenicol or rifampin had little effect on the penetration and binding of MVL51 DNA (61), indicating that neither viral transcription nor synthesis of new proteins is required for DNA penetration.

Intracellular Replication

The mode of replication of the viral DNA once it penetrates the cell membrane is known for MV-L1 only (Fig. 5). Replication of the viru

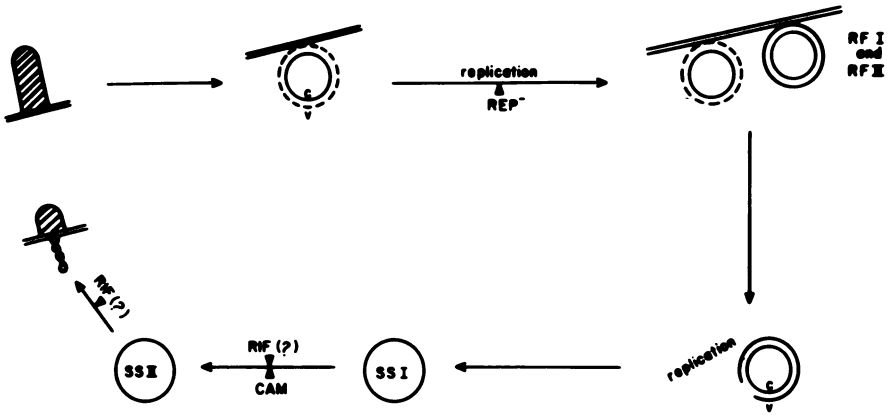


FIG. 5. *Schematic drawing of replication of MV-L1, according to Maniloff et al. (179). Broken lines show parental viral DNA strands and continuous lines show progeny DNA strands. Steps blocked by rifampin (RIF), chloramphenicol (CAM) and a REP⁻ cell variant are shown. Viral and complementary DNA strands are marked "v" and "c," respectively. The parallel double lines denote cell membrane.*

follows a similar pattern to that of the nonlytic filamentous phages (71, 179). The parental single-stranded DNA, while attached to the inner side of the plasma membrane, is converted to covalently closed double-stranded circular replicative form (RFI) and a derivative nicked form (RFII). The membrane-associated parental RF replicates semiconservatively to produce a pool of progeny RF molecules. This step requires some cellular functions, since a host cell variant, REP⁻ phenotype (218), is incapable of this step of DNA replication. The RFII molecules serve as precursors for the synthesis of single-stranded viral DNA (SSI), which involves asymmetric replication and probably takes place in the cytoplasm. Rifampin has no effect on any of the stages of viral DNA replication (61); hence, virus-specific transcription is not required for this process.

SSI is converted to SSII by the association of two of the viral proteins (molecular weights, 70,000 and 53,000). As would be expected, chloramphenicol inhibits SSII formation (61). The SSII is a short-lived intermediate which can be detected only under conditions of slow cell growth and viral replication (60). Interestingly enough, rifampin, which does not inhibit the replication of the viral DNA at the early stages of infection, blocks the conversion of SSI into mature particles (61, 180). It appears that since the RNA polymerase of uninfected *A. laidlawii* is resistant to rifampin, this antibiotic, when added late in the infection cycle, inhibits the synthesis of some virus-specific RNA. Virus-specific transcription, therefore, is necessary for the assembly step of the virus. As the mature virion contains four proteins, as against two in SSII, the final stage in MV-L1 replication involves the completion of particle assembly and its extrusion through the cell membrane. The replication processes occurring in cells infected with MV-L2 or MV-L3 are essentially unknown.

Virus Liberation

Most, if not all, bacteriophages are released after lysis of their host bacteria, an essential step in overcoming the cell wall barrier. With the wall-less mycoplasmas, virus release may be gradual, resembling the release of viruses from infected animal cells. The early observations of Gourlay (112) and of Liss and Maniloff (167) that the plaques produced by MV-L1 and MV-L2 on *A. laidlawii* lawns are turbid provided the first indication that lysis of the host cells does not occur. Growth curves of MV-L1 showed a short latent period of about 10 min followed by a gradual increase in virus titer over several hours (169), suggesting continual viral production and release. A plateau was reached about 2 h after infection, by which time about 150 to 200 virus particles had been released per infected cell. Lysis of the infected cells by 0.2% Triton X-100 did not increase the number of released viruses throughout the replication cycle, suggesting that there is no intracellular pool of completed viruses and that virus maturation and release are simultaneous. Electron microscopy showed that MV-L1 assembly and release occur at a limited number of membrane sites per cell and that clusters of extracellular progeny virus are seen at these sites (169, 206).

One-step growth and artificial lysis experiments on MV-L2 infected cells indicated that infection with this virus is also not lytic (230). Thin sections suggested that release of MV-L2 occurred by budding, and freeze-fracturing revealed that the membrane fracture faces at the budding area have fewer particles than are found

on the rest of the cell membrane fracture faces (R. M. Putzrath and J. Maniloff, personal communication), suggesting a virus-induced modification of specific membrane sites. The increased osmotic stability of *A. laidlawii* infected with MV-L2 (230) may be associated with these changes in the cell membrane. MV-L2 is the only reported nonlytic enveloped virus of procaryotes, resembling the enveloped nonlytic viruses of animal cells. It thus appears that most cells infected by MV-L1 or MV-L2 release virus gradually and do not lyse. The cell membrane apparently reseals spontaneously after the extrusion of the virus particle. Growth of the infected cells is retarded, however, and the size of their colonies is markedly reduced. Hence, the turbid plaques produced by these viruses represent areas with much smaller colonies than those produced by the surrounding uninfected cells. The picture may be different for MV-L3, as the plaques produced by this virus are small and clear, and infection is characterized by the intracellular accumulation of mature virus particles, leading to the death of the host cells (166).

Spiroplasma Viruses

Although all the spiroplasmas isolated so far show clear morphological evidence for infection with one or more types of viruses (Table 4), only one of these viruses (SV-C3) has been shown to produce plaques on *S. citri* lawns (47). Therefore, almost all of our information on spiroplasma viruses is based on morphological studies. The idea that plant and insect mycoplasmas could be infected by viruses was first suggested by observations of thin sections from infected plant and insect tissues (see literature cited in reference 47). In these cases, it was difficult to rule out the possibility that the virus-like particles observed were not derived from the plant or insect cells. Only after the first successful cultivation of spiroplasmas did it become clear that these mycoplasmas are indeed infected with viruses (48–50). In retrospect, solid evidence for viruses infecting a spiroplasma was obtained by Oishi and Poulson as early as 1970 (219), working on the sex ratio organism infecting *Drosophila*, later identified as a spiroplasma (353).

S. citri is infected by three morphologically different viruses, SV-C1, SV-C2, and SV-C3 (SV = *Spiroplasma* virus, C = *citri*), following the system proposed by Gourlay for the designation of *A. laidlawii* viruses (Table 4). SV-C1 is rod shaped, resembling MV-L1 to a great extent, though much longer; SV-C2 morphologically resembles type B bacteriophages, having a polyhedral head with a hexagonal shape. It has a long tail, apparently unsheathed and noncontractile. The end of the tail appears wider, suggesting a spiked base, but better resolution is needed to determine if this is indeed so. SV-C3 is polyhedral with a short tail, resembling MV-L3 but much smaller in size (47, 49). Similar to and in all probability identical with SV-C3 are the spv-1 and spv-2 viruses infecting the sex ratio organism spiroplasma (352).

Definite conclusions as to host specificity of the spiroplasma viruses must await their isolation. Electron microscopy revealed (47) SV-C3 infection in all the spiroplasmas known so far, and SV-C2 particles in *S. citri* only (Table 4). Apart from experimental evidence showing spv-1 to be a DNA virus, nothing is known of the nucleic acids of the other spiroplasma virus-like particles. Obviously, most of our knowledge on the mode of replication of the spiroplasma viruses is at this time indirect and very presumptive, as it is based on morphological evidence only. No intracellular precursors or completed particles of SV-C1 have been observed in sections of infected cells, suggesting that this virus assembles near the membrane and the mature virion is immediately extruded from the cell (47). Regarding SV-C2, intracellular heads, both incomplete and filled, are commonly seen in sections of *S. citri*, suggesting a different replicative cycle from that in SV-C1 (49). The SV-C3 particle was seen attaching to the outer layer of the spiroplasma membrane with the distal tip of its tail. A peculiarity of SV-C3 is its tendency to emerge from the infected cells by budding, becoming surrounded by the host cell membrane (49, 219). This enveloped stage is a temporary one, but the way by which the infectious particles rid themselves of the host membranes is unknown. Budding and virus release of spv-1 are accompanied by loss of the helicity followed by lysis of the SRO spiroplasma (219, 352).

Carrier State and Lysogeny

Although the early report of Liss and Maniloff (167) claimed that ultraviolet irradiation induced the release of MVL51 from *A. laidlawii* carrier cells, later studies usually found this difficult to reproduce, leading to the conclusion that the mycoplasma virus carrier state is extremely stable and that the formation of spontaneous plaques is an extremely rare event. That a carrier state can indeed exist is indicated by the recent work of Putzrath and Maniloff (230) showing that *A. laidlawii* cells infected with MV-L2 reach an apparently stable virus carrier state. These carrier cells have a growth rate and osmotic fragility indistinguishable from uninfected cells. The carrier culture contains free viruses and infectious centers, and every cell in the carrier culture can give rise to a clone of either carrier cells or virus-resistant cells. Since

newly infected cells release virus at a much faster rate than do carrier culture cells, it follows that the rate of viral replication must be regulated within each carrier cell. Cole's finding of infection with one or more of the three spiroplasma viruses in every *S. citri* culture examined under electron microscopy may suggest that the existence of a carrier state is very common in *S. citri* as well, yet repeated experiments to induce virus production from these cells have failed (48–50).

Prospects for Future Research

Ever since the discovery of viruses in mycoplasma it has been hoped that mycoplasma virus research will promote studies on mycoplasma genetics. So far, this hope has not been fulfilled, but several approaches for the use of viruses in genetic studies of mycoplasmas, such as transfection of viral DNA (168), have been outlined and seem quite promising. It has also been suggested that pathogenicity and antigenic variations among mycoplasma strains can be correlated with virus infection, but at present no evidence is available to substantiate these suggestions. Introducing controlled alterations in the *A. laidlawii* membrane lipid composition and protein content might prove beneficial in studying the effects of the host cell membrane composition and physical state on virus adsorption, DNA penetration, and virus release. The advantages of mycoplasmas over animal cell systems in studying these basic problems are obvious, as discussed in detail in the sections on mycoplasma membranes. In the case of MV-L2, the effects of alterations in the host membrane composition on virus envelope composition can also be readily studied. The morphological evidence for budding of MV-L2 suggests that its envelope, like that of enveloped animal viruses, is derived from that of the host. Some evidence is already available suggesting that the virus envelope differs at least in antigenic properties from that of its host, as an antiserum to *A. laidlawii* which inhibited growth of the host mycoplasma failed to inhibit growth of MV-L2, and, conversely, an antiserum to MV-L2 did not inhibit the mycoplasma (113). More recently, MV-L2, purified by density gradient centrifugation, was found to contain only four to five proteins, which differed in electrophoretic and antigenic properties from the *A. laidlawii* membrane proteins (A. L. Watkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, G24, p. 134). These data indicate that, as with the enveloped animal viruses, the MV-L2 envelope proteins are different from the host membrane proteins, though the lipids may be similar. Studies on this subject may prove to be very rewarding.

CONCLUSIONS

The mycoplasmas constitute an extremely wide and diverse group of procaryotes, having in common the lack of a cell wall. Only part of this group, most probably a minor one, has been cultivated and characterized. The complex nutritional requirements and osmotic sensitivity of these organisms are the major factors which hamper their cultivation in vitro. The mycoplasmas are separated from all other procaryotes in a class of their own, *Mollicutes*, on the basis of the lack of a cell wall. This is also supported by the small size and low G+C content of their genome and by the peculiar properties of their rRNA's and tRNA's, usually highly conserved molecules in both procaryotes and eucaryotes. A problem still unresolved, and likely to remain so, is where to place the mycoplasmas on the evolutionary ladder: are they at the bottom of it, the descendents of the primitive protocaryotes which preceded the procaryotic-eucaryotic cell split (209, 287; Searcy et al., BioSystems, in press), or do they represent a much later evolutionary development, in which the mycoplasmas were derived from wall-covered bacteria by deletion of genetic material (216)?

The extremely simple structure and limited biochemical activity of mycoplasmas, imposed by the limited genetic information included in their small genome, have made them convenient models for studying basic problems in cell biology, particularly those concerning membrane structure and function. The facility for introducing controllable changes in the fatty acid composition of the mycoplasma membrane lipids has been exploited very effectively in studying the molecular organization of membrane lipids. These studies provided perhaps the strongest evidence in support of the bilayer configuration of lipid in biomembranes and brought into focus the importance of membrane lipid composition and its physical state on transport processes and membrane-bound enzymic activities.

The requirement of mycoplasmas for cholesterol, unique among procaryotes, has been utilized to show that it functions as a regulator of membrane fluidity during changes in growth temperature or alterations in the fatty acid composition of membrane lipids. It is still unclear, however, whether this is the sole function of cholesterol in mycoplasma membranes. Acholeplasmas, which do not require cholesterol for growth, appear capable of regulating membrane fluidity by adjustment of the chain length of the saturated fatty acids synthesized by the organisms and by selective elongation and incorporation of exogenous fatty acids. Among serum lipoproteins, those having the highest cholesterol-to-phospholipid ratio, such as LDL in hu-

man serum, are the best donors of cholesterol to mycoplasmas. Cholesterol transfer occurs during a transient contact of the lipoprotein particle with the membrane. A significant percentage of the cholesterol molecules taken up flip-flop from the outer to the inner part of the lipid bilayer in membranes of growing cells. The mechanism by which mycoplasmas control the amount of exogenous cholesterol incorporated into their membrane is still unknown. Its elucidation may throw light on the general problem of cholesterol accumulation in biomembranes under pathological conditions, as in atherosclerosis.

The first strides made to elucidate the transbilayer distribution of the various lipids and proteins in mycoplasma membranes indicate the asymmetrical nature of the membranes. Future studies will probably concentrate on characterizing the protein component of the membranes, making use of the improved methodology for the solubilization and fractionation of the hydrophobic membrane proteins. More effort should also be applied to characterizing the carbohydrate elements of mycoplasma membranes and on the search for glycoproteins, components which probably participate in the interactions between the mycoplasmas and their host cells.

In general, mycoplasmas have a truncated respiratory system lacking quinones and cytochromes and therefore are incapable of oxidative phosphorylation. The required ATP may be generated either through glycolysis, the arginine dihydrolase pathway, or, as was recently suggested, by the action of acetate kinase on acetyl phosphate, a common energy-yielding pathway in clostridia. The proposal that intracellular hydrolysis of urea in ureaplasmas serves to generate energy through the formation of an ion gradient and a membrane potential is intriguing and should be explored experimentally. Despite the relative simplicity and soluble nature of the electron transport systems in most mycoplasmas, little has been done to characterize their components. In addition, our knowledge of the intermediary metabolism and regulatory mechanisms in mycoplasmas is negligible, apart from scattered information on de novo synthesis and interconversions of nucleic acid precursors in a few *Mycoplasma* species.

Another field, still in its infancy, concerns contractile processes in mycoplasmas. These include rhythmic changes in cell shape, constrictions in cells and filaments preceding cytoplasmic division, flectional and rotary motion of spiroplasma filaments, and possibly the gliding motility of some mycoplasmas. The recent finding of an actin-like protein in *M. pneumoniae* (217) is particularly important, since it is the first report of contractile proteins in procaryotes. Again, the plastic, wall-less mycoplasmas have definite advantages over other procaryotes in studies on this exciting subject.

Animal mycoplasmas can be regarded as "membrane parasites," firmly adhering to and colonizing the epithelial lining of the respiratory and genital tracts of infected animals. They appear to be very successful parasites, as they rarely kill their host and frequently cause chronic infections which are difficult to eradicate. Mycoplasmas seldom produce highly potent toxins, and the damage they cause appears to depend on their close association with the host cell surface, so that even mildly toxic metabolic by-products, such as H_2O_2 and NH_4^+, are able to exert a toxic effect on the host cell membrane. Moreover, since mycoplasmas lack cell walls and closely adhere to the animal cell surface, the possibility of fusion of the parasite's membrane with that of its host has been raised, but experimental support for it is insufficient. Sialic acid moieties on the host cell surface serve as specific receptor sites for the pathogens *M. pneumoniae, M. gallisepticum,* and *M. synoviae.* The chemical nature of the receptor sites for other adhering mycoplasmas as well as that of the binding sites on the mycoplasma cell membrane are subjects of current research.

A new and rapidly developing field is that of mycoplasma viruses. Viruses have been propagated to date only in *A. laidlawii* and were demonstrated morphologically in several spiroplasmas. As could be expected, viruses which infect wall-less procaryotes resemble bacteriophages in some properties and animal viruses in others. The ease with which the mycoplasma membrane composition can be manipulated can be used to study the effects of membrane composition and physical state on virus penetration and release and, in the case of the enveloped mycoplasma virus, MV-L2, on the properties of the virus envelope itself. Although mycoplasma viruses have not yet been applied as tools in studies on mycoplasma genetics, their use has the potential of promoting this underdeveloped field in mycoplasma biology.

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